ISOLATION, PURIFICATION AND CHARACTERIZATION OF *Penicillium charlesii* G. SMITH EXO-ß-GALACTOFURANOSIDASE

BY

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1993

I would like to dedicate this work to the memory of my father, Gabriel Tamdem, who passed away before its completion. Thank you, Daddy, for teaching me how to believe in myself; I love you.

ACKNOWLEDGEMENTS

I am deeply grateful to Dr. John Edward Gander, chairman of my committee, for his encouragement, continued support, patience and constant effort to assist me throughout the course of these studies.

I would also like to express my sincere thanks to Dr. E.M. Hoffmann, Dr. J.F. Preston III, Dr. K.T. Shanmugam and Dr. C.M. Allen, members of my committee, for their time, assistance and encouragement.

Special thanks go to my husband, Dr. Tuekam and our children, Rosine, Sandrine and Steve, whose love, support and sacrifice contributed in achieving this work.

I also thank my mother Julienne Tamdem and my sister Horthense D. Tamdem.

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LIST OF ABBREVIATIONS

CH_2O- Total carbohydrate

DEAE- Diethylaminoethyl

CM- Carboxymethyl Sepharose

ELISA- Enzyme-linked immunosorbent assay

Gal- D-Galactose

Galf- D-Galactofuranosyl residue

Rha_p- L-Rhamnopyranose

Glc_p- D-Glucopyranose

Gal_pNAc- N-Acetyl-D-galactopyranose

Gal_p- D-Galactopyranose

IEF- Isoelectric focusing

kd- Kilo-dalton

Man- D-Mannose

Manp- D-Mannopyranosyl

NMR- Nuclear Magnetic Resonance

pPGM- Peptidophosphogalactomannan

pP_2GM^{ii}- pPGM fraction II containing 2 phosphate residues

$pP_{30}GM^{ii}$- pPGM fraction II containing 30 phosphate residues

ppm- part per million

SDS-PAGE- Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Abstract of Dissertation Presented to the Graduate School of
the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

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May, 1993

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Fungi of the genera *Penicillium, Aspergillus, Talomyces* and others produce soluble extracellular polysaccharides, glycopeptides and glycoproteins. The major extracellular polymers of *Penicillium charlesii* are glycopeptides: peptidophosphogalactomannans (pPGMs). They are composed of ß-D-(1-->5)-galactofuranosyl, mannosyl, amino acyl and variable numbers of phophodiester residues.

Penicillium charlesii produces extracellular enzymes such as phosphatases, proteases and glycohydrolases. The percentage of galactofuranosyl residues in pPGM decreases with increasing age of the culture; prior to this study, the enzyme that catalyzes galactofuranosyl hydrolysis had not been purified to homogeneity nor its properties investigated.

The focus of this research was to purify and characterize extracellular ß-(1-->5)-galactofuranosidase from culture filtrates of *P. charlesii*. It was purified 100-fold by ion-exchange and gel filtration chromatography; it contains 15% carbohydrate as mannose. Mobility of the enzyme in nondenaturating PAGE indicates 2 forms of apparent mass of 70 and 150 kd; it moves with an apparent mass of 75 kd, as a single band in SDS PAGE. The pI determined by isoelectric focusing PAGE is 4.35; the optimum pH of catalysis is 4.5. ß-Galactofuranosidase is catalytically active between 25^0 -60^0C with maximum activity at 40^0C. The enzyme retains 94% of the original activity after freezing and thawing in buffer. The enzyme catalyzes galactose release from i) 1-O-ß-methyl-D-galactofuranoside, ii) 5-O-ß-galactofuranosyl-containing peptidophosphogalactomannan and iii) 5-O-ß-oligogalactofuranosides. Galactose was not released from lactose or from p-nitrophenyl-ß-D-galactopyranoside.

The specific activity of exo-ß-galactofuranosidase on i) 1-O-ß-methyl-D-galactofuranoside; ii) pP_2GM^{ii}; and iii) $pP_{30}GM^{ii}$ were 857, 417, and 134 μM hr^{-1} mg^{-1} protein, respectively. The apparent K_m of the enzyme with these substrates is 2.6, 0.8 and 1.6 mM, respectively. Exo-ß-galactofuranosidase did not release phosphorylated galactofuranosyl residues from $pPGM^{ii}$s nor was it inhibited by D-galactose-6-phosphate, D-mannose-6-phosphate or peptidophosphomannan. The results provide the first indirect evidence showing that phosphodiesters in pPGMs have a role in protecting the polymer from exo-ß-galactofuranosidase catalyzed depolymerization.

INTRODUCTION

Microorganisms such as bacteria and fungi are noted for the production of extracellular and mural polysaccharides, glycoproteins and glycopeptides. The cell surface glycoproteins may act as receptor molecules which are involved in lectin-mediated coaggregation, or they may be involved in the process of parasite attachment and internalization (De Arrura et al., 1989; McConville and Bacic, 1990; and Abeyguwardana et al., 1990, 1991a, 1991b). Some of the extracellular polysaccharides are allergenic or toxic to humans (Daley and Strobel, 1983; Sward-Nordmo et al., 1988). Many extracellular glycoproteins are enzymatically active. Most extracellular enzymes have no cellular substrate and they appear to have evolved as "scavenger" enzymes; extracellular enzymes which have cellular substrates have been implicated in several cellular processes such as differentiation, cell wall turnover and growth.

Penicillium charlesii releases into the growth medium various extracellular saccharide-containing polymers. The most well characterized class of glycopeptides of this organism is composed of galactosyl, mannosyl, amino acyl and phophodiesters residues (Preston et al., 1969a, 1969b; Gander et al., 1974; Rick et al., 1974; Unkefer et al., 1982; Unkefer and Gander, 1990).

This class includes a heterogeneous group referred to as peptidophospho-galactomannans (pPGMs). The percentage of galactose in pPGMs decreases as the culture ages; this suggests the presence in the growth medium of enzymes hydrolyzing ß-D-(1-->5) linked galactofuranosyl residues.

Many extracellular glycoproteins produced by this fungus are hydrolytic enzymes such phosphatases, proteases and glycohydrolases. Although several investigators have studied the chemical composition and structures of extracellular glycopeptides from *Penicillium* and *Aspergillus*, (Bardalaye and Nordin, 1977, Barreto-Bergter et al., 1981; Unkefer et al., 1982; Bonetti et al., 1990 and 1991), the function of these glycopeptides is still not understood. However, a likely, but yet unproven role is that of a temporary storage form of phosphate, carbohydrate, amino acid and phosphatide components (Gander and Laybourn, 1981). Salt (1983), in his studies on extracellular glycoprotein enzymes from *Penicillium charlesii*, proposed that these glycopeptides may represent cleavage or degradation products of exocellular glycoproteins. This hypothesis is consistent with the role for the glycopeptide in the post-translational translocation of glycoprotein enzymes across the cell membrane and cell wall of *P. charlesii*. While some evidence was presented in support of this hypothesis, further testing is needed to demonstrate the relationship between the glycopeptides and the extracellular glycoproteins. Isolating an

extracellular glycoprotein to apparent homogeneity and characterizing it should provide clues to the relationship, if any, of glycopeptide and extracellular glycoproteins. Also, understanding the role of enzymes that catalyze depolymerization of these extracellular glycopeptides is fundamental in determining the roles of the abundant phospho-glycopeptides; this may also help in determining how they participate in the competitive process which allows fungi to thrive in diverse environments.

In an attempt to determine the role of enzymes that catalyze the depolymerization of pPGM, we have recently focused our research on the purification and characterization of exo-ß-galactofuranosidase. Exo-ß-Galactofuranoside-containing polymers are immunodominant for some *Penicillium* and *Aspergillus spp* (Preston et al., 1970; Bennett et al., 1985; Notermans et al., 1988; Tuekam, 1991). Toxin produced by *Helminthosporium sacchari* is a ß-D-(1-->5)-linked galactofuranoside-containing sesquiterpenoid (Livingston and Scheffer, 1981). Understanding the extent of the activity of ß-D-galactofuranosidase in the depolymerization of ß-D-galactofuranoside containing-polymers is necessary in order to prevent any false results in assays intended to detect these polymers.

An endo ß(1-->5) D-galactofuranase from *Penicillium oxalicum* which specifically hydrolyzes (1-->5) linked ß-D-galactofuranosyl residues in homo- and heterogalactan has been reported (Reyes et al., 1992). An exo ß-D-

galactofuranosidase isolated from a crude commercial preparation of *Trichoderma harzanium* was purified and characterized by Van Bruggen-Van Der Lugt et al. (1992). *Penicillium charlesii* also produces a wide variety of enzymatically active extracellular glycoproteins, including acid phosphomonoesterase, alkaline phosphomonoesterase, choline specific phosphodiesterase, bis-(phenyl)-phosphate phosphodiesterase, N-acetyl-ß-glucoaminidase and α and ß glucosidase (Salt, 1983; Abbas, 1987). Salt (1983) has proposed that pPGM may be derived from these and other extracellular enzymes by proteolysis. This research provides some insight into this hypothesis.

OBJECTIVE

The purpose of this research is to determine the role of *Penicillium charlesii* exo-ß-galactofuranosidase in the depolymerization of pPGMs. The purified galactofuranosidase will also be used to determine if there is any similarity between this enzyme and pPGM.

Specific objectives are to

1-Isolate, purify and characterize exo-ß-galactofuranosidase

2-Determine general properties of exo-ß-galactofuranosidase such as pH and temperature for activity, pI , substrate specificity and kinetic constants.

3-Determine the ability of the purified enzyme to catalyze hydrolysis of phosphorylated glycopeptide.

4-Determine if exo-ß-galactofuranosidase will cleave phosphorylated galactofuranosyl residues.

REVIEW OF LITERATURE

Microorganisms such as bacteria and fungi often release a large variety of polysaccharides, glycopeptides and glycoproteins into the culture medium. These polymers often contain more than one species of sugar; mannose and galactose are common major component sugars. Frequently one or more of these sugars are derivatized with a phosphoryl or an acyl component. Some of the glycoproteins are enzymatically active. The first section of this review of literature will focus on the distribution and composition of extracellular and/or cell wall polysaccharides and glycoproteins containing galactofuranosyl residues and the second section will focus on glycohydrolases.

Galactofuranosyl-Containing Extracellular or Cell Envelope/Wall Glycoproteins and Polysaccharides

Bacteria

Carbohydrate components of bacterial polysaccharides, glycoproteins, glycolipids and proteoglycans associated with cell envelopes have a complex structure. They are usually highly branched and feature a variety of chemical modifications including acyl, ketal, phosphoryl and sulfuryl groups attached to hexosyl, pentosyl or polyol sugars.

One of the first bacterial polysaccharides shown to contain galactofuranosyl residues was obtained from *Pneumococcus* Type 34(41) (Roberts, et al., 1963). They isolated a phosphorus-free pentasaccharide by alkaline hydrolysis; the pentasaccharide was characterized as O-D-galacto-furanosyl-(1-->3)-O-α-D-glucopyranosyl-(1-->2)-O-D-galactofuranosyl-(1-->3)-O-α-D-galactopyranosyl-(1-->2)-ribitol. Plackett and Buttery (1964) isolated 6-O-ß-D-galactofuranosyl-D-galactose from *Mycoplasma mycoides* following treatment of a galactan obtained from that organism with dilute mineral acid. Since these early investigations, galactofuranosyl-containing saccharides have been isolated from several genera of bacteria.

Cell wall polysaccharides of certain oral *Streptococcus* spp. are postulated to be receptor molecules for galactose and N-acetylgalactosamine reactive fimbrial lectins of *Actinomyces* spp. Abeyguwardana et al. (1990, 1991a, 1991b) have demonstrated by selective chemical degradation, methylation analysis, mass spectrometry and ^{1}H- and ^{31}P-NMR spectroscopy that *Streptococcus* spp. cell wall polysaccharides consist of linear polymers of hexasaccharide repeating units joined by phosphodiester bonds. The repeating unit varies from one species to another.

S. oralis ATCC 10557 hexasaccharide repeating unit consists of [-->6) Galp (α-1-->3) Rhap(ß-1-->4) Glcp(ß-1-->6) Galf(ß-1-->6) Galp(ß-1-->3) GalpNAc(α1--> PO_4] (Abeygunawarda et al., 1991a). *S. oralis* C104

hexasaccharide repeating unit is composed of [-->6) Galf(ß-1-->3) Galp(ß-1-->6) Galf(ß-1-->6) GalpNAc(ß-1-->3) Galp(α-1-->1) Ribitol(5--> PO_4] (Abeygunawarda et al., 1991b).

S. sanguis J22 repeating unit is a heptasaccharide containing :[-->6) α-D-GalNAp (1-->3)-ß-L-Rhap (1-->4)-ß-D-Glcp (1-->6)-ß-D-Galf (1-->6)-ß-D-GalpNAc (1--3) α-D-Galp (1-->PO_4], with α-Rhap attached (1-->2) to ß-L-Rhap (Abeygunawarda et al., 1990). Comparison of these structures suggests that the similar lectin receptor activities may depend on the internal galactofuranosyl residue linked ß-(1-->6) to Gal (ß-1-->3)GalNAc(α) or GalNAc (ß-1-->3) Gal(α).

Lipopolysaccharide of *Actinobacillus pleuropneumonae*, causative agent of contagious pleuropneumonia in swine, was shown by chemical and NMR methods to be a unique linear unbranched homopolymer containing exclusively 1-->2 linked ß-D-galactofuranosyl units (Perry, 1989).

Capsular polysaccharides of *Klebsiella* serotype K12 and K41 were shown to contain, respectively, two and one galactofuranosyl residues (Beurret et al., 1989). O-Antigen side chain polysaccharides in the lipopolysaccharides of *Klebsiella* serotype O2 are identified as a repeating unit structure which consist of a disaccharide of 3-ß-D-galactofuranosyl-(1-->3)-α-galactopyranosyl. This repeating unit is identical to that of D-galactan present in a lipopolysaccharide of *Klebsiella pneumoniae* serotype O1 (Whitfield et al., 1992).

Mycobacterium spp cell wall arabinogalactan is covalently linked to both peptidoglycan and high molecular weight branched mycolic acids. This arabinogalactan constitutes approximately 35% of the cell wall mass (McNeil et al., 1987). It has been demonstrated that arabinogalactan contains an octasaccharide repeating unit which consists of six arabinofuranosyl residues predominantly linked (1-->5) and two galactofuranosyl residues linked (1-->5) or (1-->6), by a complex series of reactions involving methylation, partial hydrolysis, sodium borodeuteride reduction, ethylation, total acid hydrolysis, acetylation gas chromatography and mass spectrometry (McNeil et al.,1987; Gruber and Gray, 1990; and Daffe et al., 1990).

Protozoa

Several galactofuranosyl containing high mannose type oligosaccharides present in glycoprotein of many protozoa have been described (Gonzales Clemente et al., 1990, Mendelson and Parodi, 1986). In eucaryotes, oligosaccharides are processed as protein-linked glycans. Protein glycosylation in most eucaryotes are initiated by the transfer of Glc_3-Man_9-$GlcNAc_2$ from a dolichol diphosphate derivative to asparagine residues on the polypetide chains. Protein N-glycosylation in trypanosomid protozoa differs by the fact that, the transferred oligosaccharides are nonglucosylated and contain galactosyl residues (Gonzales Clemente et al., 1990). In trypanosomatid (parasitic protozoa)

Leptomonas samueli and *Herpetomonas samuel pessoai*, the carbohydrate components of glycopeptides appeared to consist of Galf, Man, N-acetyl glucosamine in various ratios; Galf is linked to mannose at nonreducing ends of the transferred oligosaccharides.

Glycoproteins of *Crithidia fasciculata* and *Crithidia harmosa* also contain galactofuranosyl residues added to the nonreducing end of mannosyl residues. These galactofuranosides are added after removal of a single mannose unit from one of the two mannoses originally present in the transferred oligosaccharide (Mendelson and Parodi, 1986).

Cell surface glycoproteins of trypomastiges of *Trypanosoma cruzi* contain ß-D-galactofuranosyl residues which are involved in the attachment and/or internalization of the parasite (De Arruda et al., 1989).

Lipophosphoglycan (LPG) of *Leishmania spp.* has a tripartite structure, consisting of a phosphoglycan, a variable phosphorylated hexasaccharide glycan core, and a lysoalkyl-phosphoinositol (lysoalkyl-Pi) lipid anchor. One galactose of the glycan core is in the furanosyl configuration (McConville and Bacic, 1990, McConville et al., 1990).

Fungi

Fungi are noted for their ability to produce extracellular and mural glycoproteins and glycopeptides. The carbohydrate components consist primarily of mannans, phosphomannans, galactans, galactomannans and glucans.

Extracellular glycopeptides of *Ascobolus furfuraceus*, with a molecular mass of about 20 kd, contain mannose, galactose, glucose and glucosamine; the molar ratio of each of these sugars varies with the media composition. The variability in the amount of galactose, present as terminal furanosyl unit, may reflect the action of an exo-ß-galactofuranosidase (Groisman and Lederkremer, 1987). Evidence of two types of linkage between the carbohydrates and the peptide moieties was provided by cleavage of the sugar chain by endo ß-N-acetyl-glucosaminidase and by alkaline treatment (Groisman and Lederkremer, 1987). Alkaline treatment of these extracellular glycopeptides results in an increase in absorbance at 240 nm with concomitant decrease in threonine and serine content.

ß-Galactofuranosyl residues have also been described in the proteogalactomannan from *Neurospora crassa*. These ß-D-galactofuranosyl units are linked to the C_2 of a mannosyl residue in O-glycosidic and N-glycosidic linkages of the peptides (Nakagima et al., 1984).

Helminthosporium sacchari, the causative agent of eyespot disease of sugar cane, produces helminthosporoside which is a toxin. This toxin, purified by thin layer, ion exchange and gel chromatography, was shown to contain an oligosaccharide composed of ß-(1-->5)-galactofuranoside units. This oligosaccharide is linked to a sesquiterpenoid $C_{15}H_{22}$ (Livingston and Scheffer, 1981). Regulation of toxin production in the culture medium containing the

fungus is in part controlled by ß-galactofuranosidase produced by the fungus (Daley and Strobel, 1983).

Cladosporium herbarum produces a glycopetide allergen Ag-54. The carbohydrate moiety of this glycoprotein is similar to the one from *Penicillium charlesii* described by Gander and coworkers (Preston et al., 1970). The carbohydrate component of the *Cladosporium* polymer is a highly branched galactoglucomannan with a molecular mass of 19 kd. Mannosyl residues are linked α-(1-->2) and α-(1-->6), while glucosyl residues are linked (1-->4) and (1-->6). The (1-->6) linked galactofuranosyl side chains are linked to the C_2 of the (1-->6) linked mannosyl units (Sward-Nordmo et al., 1988). The reducing end of mannosyl residues are O-linked to threonyl residues of a peptide.

The yeast form of *Cladosporium wernecki* also produces a similar variety of mannosyl rich, galactofuranosyl containing peptidophospho-galactomannan (Lloyd, 1972). *Trichophyton mentagrophytes* elaborates a variety of glycopeptides of molecular mass 30-40 Kd. These glycopeptides contain primarily mannosyl residues (65-75%) with nonreducing terminal galactofuranosyl residues (9-20%). The carbohydrate components are linked to a peptide rich in threonine, serine, proline and glutamine residues (Barker et al., 1967).

Cladosporium flavum extracellular glycopeptides consist of a phosphorylated glucogalactomannan which is O-glycosidically linked to a peptide rich in serine, threonine, asparagine, glutamine and proline residues. Galactose, in these glycopeptides, is in the furanosyl configuration (Dow and Callou, 1979; DeWit and Kodde, 1981).

Species of *Cryptococcus* are the only fungi with capsular polysaccharides (Bhattacharjee et al., 1984). *Cryptococcus* polysaccharides are composed of xylose, mannose, galactose and uronic acids. *Cryptococcus neoformans* cell wall polysaccharides are characterized as glucurono-xylomannan, galactoxylomannan and mannoprotein. Galactoxylomannan is composed of mannose, galactose and xylose. Galactofuranosyl units occur only in some types and are always present as non-reducing termini (James and Cherniak 1992).

The major cell wall polysaccharides of *Aspergillus fumigatus* are characterized as peptidogalactomannans (Azuma et al., 1968) and galactomannans (Sakaguchi et al., 1969; Bennett et al.,1985). The peptidogalactomannan has a mannan backbone composed of α-(1-->6)-linked mannopyranosyl residues to which are attached α-(1-->2) and α-(1-->6) linked mannopyranosyl residues, D-galactofuranosyl residues and glucosamine (Azuma et al., 1971). The galactomannan consists of a branched core

containing α(1-->2)- and α-(1-->6)-linked mannose units with linear side chains of ß-(1-->5)-galactofuranosyl and/or ß-(1-->4)-galactopyranosyl units which are terminated by galactofuranosyl nonreducing end units (Bennett et al., 1985; Latge et al., 1991; Stynen et al., 1992).

Aspergillus niger peptidogalactomannans consist of a core with α-(1-->6)-linked mannopyranosyl residues substituted at C_2 with oligomannosides and at C_6 with (1-->5)-linked ß-D-galactofuranosyl units; the average length of the galactofuran chains is 4 residues (Barreto-Bergter et al., 1981). Cell wall galactomannans of *Aspergillus niger* consist of repeating units, with 2 to 5 mannopyranosyl residues joined by α-(1-->2)-glycosidic linkages, connected by α-1-->6 linkages between each manno-oligosaccharide unit. The manno-pyranosyl residues are substituted at C_2 with a tri- or tetra-saccharide of galactose with the general structure ß-D-galactofuranosyl-(1-->4)-(galacto-pyranosyl)$_{1-2}$-(1-->4)-galactopyranosyl-1--> (Bardalaye and Nordin, 1977).

Clutterbuck and coworkers (1934) first demonstrated that culture filtrates of *Penicillium charlesii* G. Smith contain two extracellular carbohydrate polymers, galactocarolose and mannocarolose, separable by fractional precipitation with ethanol. Galactocarolose, a homopolymer of galactosyl residues, is exceptionally labile to mild acid hydrolysis. Haworth et al., 1937, showed by methylation and other chemical analysis that

galactocarolose contains 9 to 10 5-O-ß-D-galactofuranosyl residues and that mannocarolose contains four 2-O-α-D-mannopyranosyl residues attached through a (1-->6) linkage to another four 2-O-α-D-mannopyranosyl unit.

Investigations of the structure and biosynthesis of mannocarolose and galactocarolose by Gander and coworkers in the 1960s revealed that both are degradation products of a large phosphorylated extracellular precursor polymer (Gander, 1960; Preston and Gander, 1968; Preston et al., 1969a, 1969b). This polymer was later shown to be a peptidophosphogalactomannan (Gander et al., 1974). These extracellular polysaccharides and glycopeptides are believed to be primarily derived from glycoproteins, cell walls and/or cytoplasmic membrane-bound lipo-glycoproteins.

Glycopeptides of *P.charlesii*, characterized as peptidophosphogalacto-mannans (pPGMs), are protein-carbohydrate complexes with a molecular weight in the range of 25 to 70 kd (Preston et al., 1969a, 1969b; Gander et al., 1974; Rick et al., 1974; Rietschel-Berst et al., 1977). These pPGMs are heterogeneous and are isolated from culture filtrates by filtration, precipitation and anion exchange chromatography. The two major fractions, $pPGM^{ii}$ and $pPGM^{iii}$ (Salt and Gander, 1985), are composed of a mannan backbone with approximately 80 mannopyranosyl residues linked to one another primarily through α-(1-->2) and α-(1-->6) glycosidic bonds (Gander et al., 1974;

Rietschel-Berst et al., 1977; Unkefer and Gander, 1990). Reducing terminal mannosyl residues of the mannan are O-linked to seryl or threonyl residues of a peptide with 30-32 amino acyl units.

The peptide is rich in serine, threonine, alanine, glycine and proline with almost no aromatic or sulfur containing amino acids (Rick et al., 1974). These pPGMs also contain 12 manno-oligosaccharides, each with 1-3 residues, attached to the peptide, 10-20 galactofuran chains with 2-10 5-O-ß-D-galactofuranosyl residues attached to the mannan backbone and a variable number of phosphodiesters. Salt and Gander (1988) showed that the phosphate content of the major pPGM species varies with the growth medium; $pP_{30}GM$ has 30 phosphodiesters per molecule whereas $pP_{10}GM$ and pP_2GM have only 10 and 2, respectively. Both the mannan and galactan of $pP_{30}GM$ are highly substituted with phosphodiesters bridged to the C-6 hydroxyl group of the mannosyl or galactofuranosyl residue (Bonetti et al., 1990, 1991). Approximately 10 phosphodiesters, primarily N-methyl-phosphoryl ethanolamine, phosphoryl ethanolamine, phosphoryl choline are attached to the mannan backbone (Salt and Gander, 1985). Galactan chains of $pP_{30}GM$ contain approximately 20 phosphodiester residues. These phosphodiesters consist of N-peptidyl-ethanolamine of various sizes and phosphoryl choline (Bonetti et al., 1990).

Beachy (1977) isolated a galactofuranosyl-containing glycopeptide associated with the vacuolar or cytoplasmic membrane of *P. charlesii*. This glycopeptide had a molecular weight of 105,000 as determined by SDS-PAGE. Sixty percent of the carbohydrate portion consisted of galactose in the furanosyl configuration and the remaining was mannose in the pyranosyl configuration. The amino acid composition showed that serine and threonine accounted for approximately 38% of the amino acid content of the peptide. This peptide is rich in alanine, aspartic acid/asparagine, glutamic acid/glutamate, glycine and valine, and has no aromatic or sulfur containing amino acids. Furthermore, the glycopeptide contained ethanolamine and phosphate residues. The glycopeptide was deoxycholate-soluble and its lipophilic character was attributed to a covalently linked sphingolipid base tentatively identified as dihydrosphingosine.

Galactose content in pPGM decreases as the age of the culture increases (Preston et al., 1969a). The organism also secretes an exo-ß-galactofuranosidase which hydrolyses ß(1-->5)-linked galactofuranosyl residues of pPGM (Rietschel-Berst et al., 1977). The activity of the enzyme in the medium accounts for the decrease in the galactose content of pPGM as the culture ages. It was shown that 2-deoxy glucose inhibits secretion of the enzyme but not the secretion of pPGM (Gander and Fang, 1974). These data suggest that the enzyme is a glycoprotein containing mannosyl or glucosyl residues.

Rietschel-Berst et al. (1977) reported the isolation and the partial purification of an exo-ß-D-galactofuranosidase from Penicillium charlesii. This purification was achieved by affinity and gel permeation chromatography, using ß-D-galactofuranosyl residues of pPGM as affinity ligand. The enzyme catalyzes the release of galactose from methyl- and ethyl-ß-D-galactofuranoside, from 6-O-ß-galactofuranosyl-D-galactose and from 5-O-ß-D-galactofuranosyl containing peptidophospho-galactomannan. This enzyme was not active on methyl-α-D-galactofuranosides or on the methyl-D-galactopyranosides. It was later shown that the enzyme was contaminated with other glycohydrolases and phosphodiesterases.

Pletcher et al. (1981) studied factors affecting the appearance of exocellular exo-ß-D-galactofuranosidase in culture medium of *P. charlesii* and they concluded that the galactofuranosidase activity appeared in the medium only after the medium is depleted of glucose and when the pH of the medium is above 4. They also suggested that galactofuranosidase activity was destroyed by an acid protease. It was shown later that when plates containing *P. charlesii* have been stored at room temperature for longer than 2-4 weeks, inoculum from these cultures showed acid protease release into the growth medium (Abbas, unpublished).

Van Bruggen-Van Der Lugt et al. (1992) have demonstrated, in addition to terminal and internal ß-(1-->5)-linked galactofuranosides, ß-(1-->6) and ß-(1,5,6)-linked galactofuranosides in *Penicillium* and *Aspergillus* species using a purified exo-ß-galactofuranosidase from *Trichoderma harzianum*.

Glycohydrolases

Many microorganisms produce extracellular enzymes. The majority of extracellular enzymes are depolymerases acting on polysaccharides, proteins and nucleic acids; hydrolases constitute the majority of these enzymes, although, the action of hexuronosyl lyases are well known also. These extracellular enzymes have no cellular substrate and seem to have evolved as scavenger enzymes which degrade polymeric material in the environment to provide the organism with assimilable nutrients. Polysaccharides degrading enzymes have a long history of commercial application in food processing, horticulture, agriculture and protein research.

Among bacteria, *Bacillus* and *Clostridium spp.* are prolific producers of extracellular glycohydrolase enzymes. Filamentous fungi and yeasts also secrete a variety of these extracellular enzymes.

Cellulases

Cellulases are enzymes which hydrolyze ß-(1-->4) glycosidic bonds in crystalline and/or amorphous cellulose, and in water soluble cellulose derivatives such as carboxy-methyl or hydroxy-methyl celluloses. Cellulases are inherently interesting. The study of cellulase initially focused on the means of inhibiting their degradative action on cellulolytic material. More recently, cellulases have gained an economic interest as they are prime agents of decay and also the potential key to conversion of waste biomass into fermentation feedstocks.

Three major types of enzymes are found in cellulase systems that can degrade crystalline cellulose. They are: endo-ß-(1-->4)-glucanase which hydrolyzes cellulose at random to produce oligosaccharides with different degrees of polymerization, exo-ß-(1-->4)-glucanase which acts by removing glucose or cellobiose from the nonreducing end of the chain and ß-glucosidase which hydrolyzes cellooligosaccharides to release glucose (Wood and McCrae, 1982; Reese et al., 1976; Wood et al., 1980; Bhat and Wood, 1992).

Extracellular cellulases have been purified from a number of bacterial species such as *Clostridium* spp. (Thomas and Zeikus, 1988; Lamed et al., 1983; Lamed and Bayer, 1988), *Cellumonas uda* (Nakamura and Kitomura, 1988) and *Streptomyces* spp (Stutzenberger, 1991). Cellulolytic bacteria which produced only cell bound cellulase such as *Cytophoga* have been described

(Chang and Thayer, 1977). All cellulotic bacteria produce an endo-ß-(1-->4)-glucanase and either a ß-glucosidase or an exo-ß-(1-->4)-glucanase or a combination of both (Gong et al., 1977; Ohmiya et al., 1987). Cellulase systems of *Cellumonas uda* consist of a cellobiohydrolase, exo-ß-(1-->4)-glucanase and a ß-(1-->4)-endo-glucanase. The former releases cellobiose and a trace of cellotriose (Nakamura and Kitamura, 1988). Cellulose degrading enzymes of *Clostridium thermocellum* are arranged into a multisubunit complex called cellulosome, which can be extracellular or cell bound (Lamed and Bayer, 1988; Ljungdahl et al., 1988).

Some fungal cellulase systems have been investigated in detail; the most studied systems are those from *Trichoderma* spp. (McCleary and Shameer, 1987; Voragen et al., 1988; Shulein, 1988), *Aspergillus* spp. and *Penicillium* spp. (Wood et al., 1980, Stewart and Parry, 1981; McCleary and Shameer, 1987; Teunissen et al., 1992; Jun et al., 1992). Most cellulolytic fungi synthesize exo-ß-(1-->4)-glucanase, endo-ß-(1-->4)-glucanase and ß-glucosidase. It is proposed that the cellulase complex requires synergetic action of at least two components for effective hydrolysis of cellulose (Voragen et al., 1988). It is suggested that endo ß-(1-->4)-glucanase first cleaves in a random manner to produce chain ends for subsequent attacks by exo-enzymes (Reese, 1977; Wood et al., 1980).

Cellulase activity is regulated through the synthesis of one or more enzyme components, by induction and/or by catabolite repression (Yamane and Suzuki, 1988), or by end-product inhibition of the activity of a single component of the complex. Gong et al.(1977) have proposed a unified model for regulation of cellulase synthesis. This mechanism of regulation takes into account the importance of glucosidase in the regulation of glucose and cellobiose levels in cells. In this model, cellulose and glucose represent the limiting cellulotic products. Cellulose serves as a carbon source and a potential inducer of the cellulase system whereas glucose and glycerol are repressor (Steward and Leatherwood, 1976 ; Nisizawa et al., 1972; Canevascini et al., 1979). ß-Glucosidase is generally responsible for regulation of the whole cellulotic process and is often the rate limiting enzyme (Kadam and Demain, 1989).

Stutzenberger (1985) proposed a model of regulation of cellulase systems similar to the well studied ara operon of *Escherichia coli*. In this model, the cellulose operon is an activator-controlled inducible system, requiring the cooperative binding of two regulatory proteins to the control region for maximal transcription.

α-Glucanases

Amylolytic enzymes are an important group of industrial enzymes. They are conveniently divided into α and ß glucanases depending on their ability to hydrolyze α or ß glucans respectively.

Various α-glucanases from a variety of sources have different physicochemical properties and product patterns. α-Glucanases are widely distributed in microorganisms and they fall into three major categories (Fogarty and Kelly, 1980): i) exo-α-(1-->4)-amylases, ii) endo-α-(1-->4)-amylases and iii) debranching enzymes.

Exo-α-(1-->4)-amylases can be further classified as glucoamylases or amyloglucosidases, ß-amylases and α-glucosidases. Glucoamylases are exo-acting amylases that catalyze hydrolysis of α-(1-->4) and α-(1-->6) linkages of glucans to produce glucose. The relative rate of hydrolysis depends on the enzyme source and on the linkages in the vicinity of the bond being hydrolyzed. Glucoamylases occur primarily in fungi and are less frequent in bacteria and yeasts (Singh and Agrawal, 1981). *Aspergillus* "amylase" was shown by Ueda and Saha (1983) to be a glucoamylase. Fungal glucoamylases can be divided into two groups depending on their efficiency of hydrolyzing starch. The first type of fungal glucoamylase hydrolyzes starch completely to glucose such as *Rhizopus delemar* glucoamylase. Hiromi and coworkers (1973) have shown that this enzyme which catalyzes the depolymerization of

maltooligasaccharides, displays an intrinsic rate of hydrolysis which is constant over a range of substrate containing 2 to 7 4-O-α-D-glucosyl residues, and that the subsite affinities increase in an additive manner as the degree of polymerization increases from 2 to 5. The other type of fungal glucoamylases hydrolyzes only 80% of the the starch; glucoamylase from *Aspergillus niger* falls in this category.

ß-Amylases hydrolyzes α-(1-->4)-glucans by sequentially removing maltosyl units from nonreducing terminal ends. Such enzymes have been isolated from *Bacillus* spp. (Fogarty and Griffin, 1975; Murao et al., 1979).

α-Glucosidases catalyze the hydrolysis of nonreducing end α-(1-->4) glucosyl residues; glucose is released. Major producers of α-glucosidases are *Aspergillus* spp. and *Bacillus* spp.

Endo α-(1-->4)-amylases are enzymes that randomly hydrolyze α-(1-->4) bonds in an endo-manner, but bypass α-(1-->6)-linkages in amylopectin and glycogen to produce a variety of oligosaccharides (maltose, maltotriose and some glucose). The rate of hydrolysis of α-(1-->4)-linkages depends on the degree of branching. Endo α-(1-->4)-amylases are produced by a wide range of microorganisms including *Bacillus* spp., *Aspergillus* spp. and *Penicillum* spp. Debranching enzymes--isomaltases and pullulanase--are enzymes whose primary specificity is to hydrolyze α-(1-->6)-linked glucose residues. Isoamylases are produced by a wide variety of organisms such as *Cytophaga*

spp. and *Streptomyces* spp. (Gunja-Smith et al., 1970; Yagisawa et al., 1972). Pullulanases from *Bacillus* spp. and *Streptomyces* spp. cleave α-1-->6 linked glucose units of starch to produce linear dextrins (Yagisawa et al., 1972; Urlaub and Wober, 1975).

ß-Glucanases

The ß-glucanases may be classified as exo- or endo-ß-glucanases. In addition, endo-ß-glucanases may be further divided into two groups depending on whether they produce large or small oligosaccharides (Mori et al., 1977; Rey et al., 1982). The ß-(1-->2)-glucanases, ß-(1-->3)-glucanases and ß-(1-->6)-glucanases from fungi and/or bacteria have been described (Reese et al., 1961, 1962; Bacon et al., 1970; Fleet and Manner, 1977; Bielecki and Gallas, 1991; Cutfield et al., 1992). The ß-(1-->6)-glucanases from *Bacillus subtilis* catalyze release of mannan containing 3% of glucose from the alkali soluble polysaccharide fraction of yeast cell wall (Fleet and Manner, 1977). Recent studies have focused on the ß-(1-->3)-glucanases from bacteria which are important in enzymatic lysis of cell wall components of yeasts and fungi. *Streptomyces* spp. 1228 produces 4 extracellular ß-(1-->3)-glucanases. It has been shown that four ß-(1-->3)-glucanases act synergistically to lyse yeast cell walls (Bielecki and Galas, 1991). *Bacillus circulans* also produces extracellular ß-(1-->3)-glucanase that lyses fungal cell walls. The enzyme is

an endo-ß-(1-->3)-glucanase and has a random cleavage pattern (Aano et al., 1992). Exo-ß-(1-->3)-glucanase from *Candida albicans* has been crystallized (Cutfield et al., 1992).

Penicillium italicum produces at least 3 types of ß-(1-->3)-glucanases with different modes of action in addition to a ß-(1-->6)-glucanase. Type I has an endo-glucanase activity, type II has an exo-glucanase activity, whereas type III has both activities. Santos et al. (1978) have shown that type II and type III are repressed by excess glucose and that the absence of glucose not only results in an increase in synthesis of both types II and III, but also triggers the synthesis of type I. Cooperativity in cell wall depolymerization by fungal endo- and exo-glucanases has been reported by Jones et al. (1974).

Glucanases, as well as several other enzymes secreted by yeasts and fungi, are glycoproteins. Their carbohydrate moieties consist of mannose, glucose, galactose and N-acetylglucosamine. N-acetyl glucosamine is bound to asparagine of the polypeptide chain (Villa et al., 1978; Sanchez et al., 1982; Rosa et al., 1984).

ß-Mannanases

The ß-D-mannanases are enzymes that randomly hydrolyze ß-(1-->4)-mannan chain of galactomannans, glucomannans, galactoglucomannans or mannans from microorganisms and/or plants. The production of ß-

mannanases and mannosidases has been reported in fungi and bacteria (Horikoshi, 1991; Araujo and Ward 1990).

Bacteria, such as, *Bacillus* spp., *Aeromonas* spp. and *Streptomyces* spp., produce endo-ß-mannanases and/or exo-ß-mannanases. An exo-ß-mannanase (ß-(1-->4)-D-mannan mannobiohydrolase), isolated from culture fluids of *Aeromonas* spp, hydrolyzes ß-(1-->4)-D-mannosyl unit of mannan with three or more mannosyl residues and release mannobiose from the nonreducing end of these oligosaccharides. This enzyme does not act on mannan substituted with D-galactosyl (galactomannan) or with D-glucosyl (glucomannan) units. These organisms also secrete endo-ß-(1-->4) mannanase (Araki and Kitamikado, 1981).

Endo-ß-mannanase from *Bacillus* spp. hydrolyzes mannooligosaccharides larger than mannotriose and produces di-, tri- and tetra-saccharides. Dekker and Richards (1976) reported constitutive and inducible bacterial mannanases. Productions of higher mannanases activities in *Bacillus* spp. have been shown to be induced by either the carbon source or the nitrogen source (Araujo and Ward, 1990), or under alkaline conditions (Horikoshi, 1991). Inducibles ß- mannanases have also been described in *Aeromonas* spp. (Araki and Kitamikado, 1982). Fungal mannanases have been reported by several authors (Reese and Shibata, 1965; Erikksson and Winell, 1968). *Aspergillus*

niger ß- mannanases have pH optima at 3.0 and 3.8 and a temperature optimum of 65^0C (Erikksson and Winnell, 1968). *Aspergillus tamarii* produced an inducible ß-D-mannanase. This enzyme is a glycoprotein containing N-acetylglucosamine, mannose and galactose and is secreted into those growth media containing galactomannan. Hydrolysis of galactomannan, by *Apergillus niger* ß-mannanase, produces mannobiose and mannotriose. The extent of hydrolysis depends on the galactose content (Civas et al., 1984); the greater the percentage of galactose, the less the percentage of total mannooligosaccharides released.

Galactanases

Galactanases, the enzymes hydrolyzing galactan components of cell wall and extracellular polysaccharides of microorganisms and/or plants, are produced by bacteria, fungi and plants (Dekkers and Richards, 1976; Nakano et al., 1985, 1990; Tsumura et al., 1991). Three types of endo-ß-galactanases have been described so far: endo-ß-(1-->3)-galactanases, endo-ß- (1-->4)-galactanases and endo-ß-(1-->5)-galactanase.

Endo-ß-(1-->3)-galactanase from *Rhizopus niveus* has been characterized; this enzyme hydrolyzes 1,3-type arabinogalactans and releases D-galactose, several 1,3- and 1,6-linked ß-D-galactose oligosaccharides. Some of these oligosaccharides contain arabinose (Hashimoto, 1971).

A ß-D-galactanase specific for ß-(1-->5)-linked galactofuranosyl residues has been purified and characterized from culture filtrates of *Penicillium oxalicum* (Reyes et al., 1992). This enzyme is a basic glycoprotein, which hydrolyzes ß-(1-->5)-D-galactofuranosyl linkages in homo- and heterogalactans with production of mono-, di- and trisaccharides.

Endo ß-(1-->4)-galactanases are the most characterized of the galactan degrading enzymes. Galactotriose and galactotetraose are the main products of the hydrolysis of galactan by *Bacillus* spp S2 endo galactanase. *Bacillus* spp 39 endo-ß-(1-->4)-galactanase primarily releases galactobiose and galactotriose. This enzyme shows a unique property with two pH optima at 4.0 and 9.0. *Bacillus subtilis* K-50 endo-(ß-1-->4)-galactanase yielded galactotriose as the major end product. The end products of the activity of the enzyme from *B. subtilis* var *amylosacchariticus* are similar to those from *Bacillus* spp. 39 enzyme, while the products of the activity of the enzyme from *B. subtilis* (wild type) are primarily galactotetraose (Labavitch et al., 1976; Araujo and Ward, 1990). Two endo ß-(1-->4)-galactanases from *P. citrium* were found to hydrolyze ß-(1-->4)-galactoside polymers to release galactose, galactobiose and galactotetraose (Nakano et al., 1985). These two enzymes have similar physical and enzymatic properties although they are separable by affinity chromatography and by polyacrylamide gel electrophoresis.

Bacillus subtilis produced an exo-ß-(1-->4)-galactanase that was shown to hydrolyze ß-(1-->4)-galactosidic linkages with major production of galactobiose and a minor production of galactotetraose. This enzyme also has a transferase activity (Nakano et al., 1990). Araujo and Ward (1990) have reported the production of inducible galactanases in *Bacillus* spp.; galactose was the inducer.

Aspergillus oryzae, *Bacillus* spp. and *Kluyveromyces* spp. produce ß-galactosidases which hydrolyze ß-D-galactobiose,-galactotriose and galacto-oligosaccharides with release of D-galactose from the nonreducing end of the galactan chain. An exo-ß-D-galactofuranosidase was partially purified from the culture filtrates of *P. charlesii* by Rietschel-Berst et al. (1977). This enzyme catalyzes the release of galactose from methyl and ethyl ß-D-galactofuranosides, from 6-O-ß-galactofuranosyl D-galactose and from 5-O-ß-D-galactofuranosyl-containing peptidophosphogalactomannan and is not active on either 1-O-α-methyl-D-galactofuranosides or on methyl-D-galactopyranosides. Studies of factors affecting the appearance of this exo-ß-D-galactofuranosidase in the medium by Pletcher et al. (1981) showed that the enzyme is repressed by glucose and at low pH.

An exo ß-D-galactofuranosidase isolated from crude commercial preparations of *Trichoderma harzianum* was partially purified and characterized by Van Bruggen-Van der Lugt et al. (1992). This enzyme releases galactose from oligosaccharides of ß-(1-->5)-linked galactofuranosides with four or more residues and from heterogalactans.

It is apparent that galactofuranosyl-containing saccharides are widespread in nature; however, they are not considered as common constituents of oligo- or polysaccharides. Exo-ß-galactofuranosidases and galactofuranases have been isolated from a few species. Although the galactofuranase was purified to homogeneity, the exo-ß-galactofuranosidases have not been purified to homogeneity. There have been no reports of either exo-α-D-galactofuranase or exo-α-D-galactofuranosidase. *Penicillium charlesii* was selected as the source of exo-ß-galactofuranosidase production because of its ability to produce and secrete relatively high activities of this enzyme (Rietschel-Berst et al., 1977).

MATERIALS AND METHODS

All chemicals used were reagent grade. These chemicals were purchased from Sigma Chemical Company and Fisher Scientific. Commercial enzyme prepa-rations were purchased from Sigma Chemical Company and from Worthington Biochemical Corporation. Filter papers, membrane dialysis apparatus and filters were purchased from several sources such as Whatman, Amicon and MSI. Columns and column chromatography supplies were obtained from Pharmacia, Bio-Rad Laboratories, and Amicon. Electrophoresis equipment and reagents were purchased from Bio-Rad and Pharmacia.

General Procedures

Spectrophotometric measurements at wavelengths between 280 nm and 820 nm were made using 1 cm path length quartz cuvettes in a Hewlett Packard Model A Diode Array Spectrophotometer. Colorimetric assays for total carbohydrate were determined at 480 nm using a Coleman II Jr. (Perkin-Elmer) spectrophotometer. Samples of 1.3 ml were examined in 10 x 75 mm borosilicate culture tubes. The pH of the culture filtrates and buffers was determined using an Accumet pH meter 900 (Fisher Scientific) standardized with appropriate buffers. The pH was also monitored using pH paper.

Filter papers (#4, Whatman) were used in the filtration harvest of *P. charlesii* cultures. Dialysis was performed using Spectrapor membrane tubing

with standard cellulose acetate dialysis tubing of 1,000, 3,500 and 14,000 to 15,000 molecular weight nominal cut-off (Union carbide).

Enzyme preparations and buffers were filter-sterilized using a Millipore filter or MSI apparatus with a filter membrane of 0.22 micron pore size.

Organism

Penicillium charlesii G. Smith 1887 was grown on Czapek Dox (CD) agar plates containing 5% glucose and 2% agar for 2 to 3 weeks at room temperature and stored at 4^0 C until use. Transfers were made every 3 to 4 months by streaking conidiospores on fresh plates.

Culture Conditions and Growth Media

Conidiospore suspensions in sterile solution containing 0.9% NaCl (w/v) and 0.125% Tween 20 (w/v) were used for inoculation of liquid shake cultures according to the following procedure: 2 ml of spore suspension ($\sim 10^6$ conidiospores), were diluted in 150-200 ml sterile growth medium in a 500 ml notched wide mouth Erlenmeyer flask. Each flask was plugged with a styrofoam disc and agitated on a New Brunswick Model G-10 Gyrotory shaker at a setting of 10, (40 rpm). Liquid shake cultures were grown under constant light at 20^0C. Media composition is listed in table 8 and 9 (Appendix).

Isolation of Glycopeptides

Six days after inoculation, the cultures were filtered through Whatman # 4 filter paper. The filtrate was dialyzed at 4^0 C against several changes of distilled water. Peptidophosphogalactomannans (pPGMs) were isolated according to the procedure described by Salt (1983). The procedure followed is outlined in scheme I, (Appendix). After fractionation of pPGM on DEAE cellulose, fraction 2 and 3 ($pPGM^{ii}$ and $pPGM^{iii}$), were recovered and stored separately as lyophilized powders.

Chemical Modifications of Glycopeptides

Dilute acid hydrolysis of pPGM with release of galactose

This procedure involved the release of galactofuranosyl residues of pPGM using mild acid conditions. The ratio of galactose concentration to that of HCl is critical depending on whether total or partial hydrolysis is desired. For the conversion of galactan to galactose, a sample of pPGM containing approximately 2 μmol of hexosyl residues was treated with 10 ml of 0.01 N HCl for 90 min at 100^0C. The reaction was terminated by neutralization with 1 N NaOH. Following this procedure, the low molecular weight substances were removed by dialysis in cellulose acetate membranes (3500 MW cut-off). The retentates were assayed for carbohydrate, protein, and phosphate.

Preparation of oligosaccharides from pPGMs

Oligosaccharides were obtained from pPGMs by mild acid hydrolysis. Four hundred micrograms of pP_2GM^{ii} (2.5 μmoles of hexosyl residues) was treated for 20 minutes at 100° C with 1 ml of 0.01 N HCl . Dialysates (less than 3500 MW) were concentrated and applied to a DE 52 anion exchange column. The unbound material (washed through with water) was concentrated and applied to a Bio Gel P4 column.

Isolation of Enzymes

Exo-ß-galactofuranosidase from 18-21 day old Raulin-Thom liquid-shake cultures of Penicillium charlesii was isolated in a 5-step procedure. Medium, from 18-20-day cultures to which phenylmethylsulfonylfluoride (PMSF) was added at a concentration of 10^{-4} M 24 hours before harvest, was filtered through a Whatman # 4 paper.

Step 1. Culture filtrates, previously dialyzed at 4^0C against several changes of 50 mM sodium citrate buffer, pH 5.0, were concentrated 10-fold in an Amicon ultrafiltration cell with a YM 30 ultrafilter (30,000 MW cut-off) and stored at 4^0 C.

Step 2. DEAE-cellulose chromatography I. A 50 ml sample of concentrated proteins equivalent to 500 ml of culture medium, from step 1, was applied at room temperature to a column of DEAE cellulose (3 X 28 cm)

equilibrated with 50 mM sodium citrate buffer, pH 5.0. The column was washed with 500 ml of the above buffer. Exo-ß-galactofuranosidase activity eluted with unbound proteins. Fractions with ß-D-galactofuranosidase activity were collected and concentrated as before to approximately 2 ml and then resuspended in up to 20 ml with 50 mM MOPS buffer, pH 7.5.

Step 3. DEAE-cellulose chromatography II. The pooled DEAE I fraction containing galactofuranosidase activity from step 2 was applied at room temperature to a column (2 X 25 cm) of DEAE cellulose, previously equilibrated in 50 mM MOPS buffer (pH 7.5). The column was washed with the 50 mM MOPS buffer (pH 5.0) to remove unbound proteins; washing continued until no absorbance was detected at 280 nm. Elution was performed with a stepwise gradient using washes of 0, 0.12, 0.25 and 0.5 M NaCl in 50 mM sodium citrate buffer, pH 5.0. Galactofuranosidase activity eluted with 0.25 M NaCl. Active fractions were pooled and dialyzed at 4^0C against 12.5 mM sodium tartrate buffer, pH 3.0.

Step 4. Carboxymethyl sepharose chromatography. Dialyzed samples from step 3 were loaded onto a CM-sepharose (2 x 26 cm) column preequilibrated with 12.5 mM sodium tartrate buffer, pH 3.0, and the column was washed with 300 ml of the above buffer. Elution was performed again with a stepwise gradient of 0 to 0.5 M NaCl in 50 mM sodium acetate buffer, pH

4.0. Exo-ß-galactofuranosidase activity eluted with 0.25 M NaCl. Fractions which showed galactofuranosidase activity were dialyzed at 4^0C against 10 mM acetate buffer, pH4.0, containing 10 mM NaCl. The dialyzed samples were filtered through a 0.22 μm filter unit (Micron Separation Inc.) and the filtrate was concentrated in a Centricon 30 microconcentrators (30,000 MW cut-off; Amicon).

Step 5. FPLC Gel filtration. A concentrated sample (200 μl) from step 4 was applied to a Superose-12 Fast Performance Liquid Chromatography column, previously equilibrated with 10 mM sodium citrate at pH 4.0 and containing 10 mM NaCl buffer. Elution was performed with the same buffer. Galactofuranosidase positive fractions were pooled, concentrated and reapplied to the same column.

Assays of Enzyme Activities

Galactofuranosidase assay

Exo-ß-galactofuranosidase activity was assayed by two different methods. Galactofuranosidase activity was determined as described by Rietschel-Berst et al., (1977). Essentially, a reaction mixture containing 200 μl of 5 mM 1-O-ß-methyl-D-galactofuranosidase, 20 to 200 μl of enzyme preparation and 66 mM sodium acetate buffer at pH 4.0 in a total volume of 500 μl was incubated at 40^0 C for various lengths of time. Similar reaction

mixtures without either substrate or enzyme were used as controls. Substrates were diluted in 66 mM acetate buffer unless stated otherwise. Galactose released by the action of the enzyme was estimated either by Nelson test for reducing sugars (Nelson, 1949) or by the coupled oxidation of galactose and o-cresol catalyzed by galactose oxidase and peroxidase, respectively, (see below). One enzyme unit is defined as the activity which releases 1.0 μmol of galactose at 40^0 C in a minute. The substrate, 1-O-ß-methyl-D-galactofuranoside, was synthesized and purified according to the method of Augestad and Berner (1954) with a slight modification (Rietschel-Berst et al. 1977). Alternatively, the activity of exo-ß-galactofuranosidase was determined by monitoring the change in optical rotation of the substrate using a Jasco DIP Digital polarimeter. For this purpose, a reaction mixture containing 5 to 10 μmol of substrate (in 400 μl volume), 200 μl of enzyme preparation and 1.5 ml of 66 mM acetate buffer, was incubated in a one decimeter cuvette. The optical rotation was monitored periodically.

Assays for Other Hydrolases

Activities of other hydrolase were determined by spectophotometric measurement at 410 nm of p-nitrophenol released by cleavage of p-nitrophenyl esters or glycosides. Enzyme activities determined by this method were: α-D-galactopyranosidase, ß-D-galactopyranosidase, N acetyl ß-D-glucosaminidase,

acid phosphatase, and phosphodiesterases (bis-p-nitrophenyl phosphate and p-nitrophenyl phosphoryl choline as substrates). Reaction mixtures contained 5 μmol of substrate in 200 μl volume, 200 μl of 66 mM acetate buffer at pH 4.0 and 100 μl of enzyme preparation. After incubating for two hours at 40^0C the reactions were stopped by the addition of 1.0 ml of 0.2 N NaOH, the absorbance at 410 nm was measured. An extinction coefficient of 18.3 mM^{-1} cm^{-1} was used.

Galactose Oxidase Assay

Galactose released by the enzyme was routinely quantitated by the method described in the Worthington Manual. Two mg of horseradish peroxidase (Sigma) was dissolved in 10 ml of 0.1 M potassium phosphate buffer, pH 8.0 with 5 μl of o-cresol and designated solution A. Two hundred μl of solution A and 50 μl of galactose oxidase (Worthington) were added to a sample volume of 0.6 ml. The above solution was incubated at 37^0C for 30 minutes. The values obtained at 410 nm were compared with a reference solution of 1 μmol galactose.

Chemical Assays

Total Carbohydrate

Total carbohydrate was determined by the phenol-sulfuric acid method of Dubois and coworkers (1956). In this procedure, samples in a total volume of 0.3 ml aqueous solution were mixed throughly after addition of 20 μl of

80% phenol and 1.0 ml of concentrated H_2SO_4. The absorbances of the preparations were determined at 490 nm and the values obtained compared with a reference solution of 0.9 mM galactose and 0.3 mM mannose.

Reducing Carbohydrate

Reducing sugars were determined by the Nelson's test (1944) for reducing sugars. Absorbance of the blue complex was determined at 600 nm.

Total Phosphate

Total phosphate was determined using the ashing technique of Ames and Dubin (1960) and the color reagent of Ames (1966). In this assay, 20-200 μl aliquots of samples were taken to dryness over an open flame after addition of 60 μl of 10% $Mg(NO_3)_2$. The above dried preparations were then hydrolyzed by adding 0.6 ml of 0.5 N HCl and placing the reaction mixture in a boiling water bath for 15 minutes. Molybdenum-ascorbate reagent (5:1, v/v) in the amount of 1.4 ml was added to the cooled preparations and the final solution was incubated at 45^0C for 20 minutes. Absorbances were then determined at 820 nm and the values obtained were compared with a reference solution of 0.4 μmol KH_2PO_4.

Protein

Protein was estimated by the micro procedure of BCA protein assay (Pierce Biochemicals). In this assay, 0.1 ml of the sample was mixed with 2.0 ml of BCA reagent and incubated for 30 miniutes at 60° C. Bovine serum

albumin (Pierce Biochemicals) at a concentration of 0.2 mg/ml was used as reference.

Formaldehyde

Formaldehyde was estimated using the chromotropic acid assay of McFadyen (1945). A solution of oligosaccharides or glycopeptides (4 to 7 μmoles anhydrohexose/ml in H_2O) was treated with approximately a 5-fold molar excess of sodium metaperiodate for 18 hours in the dark at 4^0C. After this period, the excess periodate was destroyed by addition of sodium arsenite. The oxidized sample in a total volume of 0.1 ml was then added to 2 ml of chromotropic acid solution (10 mg/ml)-12.5 N H_2SO_4 reagent (1:4, v/v), and incubated in 100^0C water bath for 30 minutes. The samples were blanked against a control lacking saccharides, but otherwise treated similarly. Their absorbances were determined at 570 nm and the values obtained were compared with a reference solution of 1.3 μmol of formaldehyde.

Chromatography

Paper Chromatography

Descending paper chromatography of monosaccharides was carried out on 56 x 22 cm sheets of Whatman No. 3 paper. Chromatography was conducted at room temperature in glass tanks lined with Whatman No. 3 paper previously saturated with the solvent employed. Development of chromatogram was carried out for 20 hours using Solvent A consisting of n-

butanol:pyridine:water, (6:4:3), (v/v/v). The bottom edge of the chromatogram was serrated, and the solvent was allowed to drip off the chromatogram.

Thin-Layer Chromatography

The dansyl derivatives of amino acids were separated and identified by two-dimensional thin-layer chromatography on polyamide layer sheets (Chen Chin Trading Co., Ltd.). Solvent A, consisting of 1.5% formic acid in water was used for developement in the first direction. Solvent B, consisting of benzene:acetic acid, 9:1 (v/v), was used for developement in the second direction.

Detection of Compounds on Paper and Thin-Layer Chromatography

Reducing sugars were detected on paper chromatograms with alkaline silver nitrate (Trevelyan et al., 1950).

Dansyl derivatives were detected on thin-layer chromatograms by illumination of the chromatogram with ultra violet light.

Ion-Exchange Chromatography

Whatman DEAE cellulose (DE-52) and CM sepharose (fast flow) obtained from Pharmacia were employed for the routine purification of the enzyme. Precycling of the ion exchanger was carried out as prescribed in the technical literature published by the Manufacturers. All ion exchangers were

washed, swollen, and/or fines removed as recommended by Manufacturers. Column packings were pre-equilibrated in buffer when appropriate. Columns were packed by filling their lower third with buffer or other appropriate solution. A deaerated slurry of packing material was added and allowed to settle to the desired height.

DEAE cellulose (DE-52) for glycopeptide isolation was equilibrated with 0.05 M borate *in situ*. DEAE cellulose (DE-52) for enzyme isolation was preequilibrated with 0.05 M sodium citrate (pH 5.0) or 0.05 M MOPS buffer (pH 7.5). CM sepharose for enzyme isolation was preequilibrated with 12 mM sodium tartrate (pH 3.0).

Gel Filtration

A Superose 12 gel filtration column (Pharmacia) was used in the final step of enzyme purification. This column has a void volume of 7.5 ml (determined with blue dextran MW = 2.0 x10^6). Enzyme preparations containing 20 to 100 μg of protein in a total volume of 200 μl of buffer (0.01 M acetate/ 0.01 M NaCl pH 4.0) were injected . The column was washed with the same buffer at a flow rate of 0.12 ml/min.

Gel Electrophoresis

SDS Polyacrylamide Gel

Polyacrylamide gel electrophoresis of exo-ß-galactofuranosidase preparation was performed using a Bio-Rad mini slab gel apparatus (Model Mini 2-D) attached to a power supply unit (Fisher biotech Electrophoresis Systems). Specialized reagents were obtained from Bio-Rad. Gels were cast in 7 x 10 cm glass plates. Buffer systems were used as described by Laemmli (1970). Separating and stacking gels contained 12% and 4% acrylamide, respectively. Samples were applied to gels in SDS sample buffer (pH 6.8, 50 mM Tris HCl containing 10% glycerol, 2% SDS, 5% mercaptoethanol, and 0.002% bromophenol blue), heated at 100^0 C for 5 minutes. The separation was achieved using constant current at 200 volts for approximately 45 minutes. The gels were removed from plates, fixed and stained with Coomassie Brilliant Blue R-250, silver stain followed by Coomassie Brilliant Blue R-250. Exo-ß-galactofuranosidase, a glycoprotein, stains poorly when stained with Coomassie or silver stain alone. The staining procedure which requires the sequential staining as indicated above gave visible bands.

Non-Denaturating Polyacrylamide Gel

Non-denaturating gel electrophoresis of exo-ß-galactofuranosidase was performed using the same apparatus as above. Buffer systems were used as

described in the Sigma technical bulletin No. MKR-137. Separating gels contained 7% to 10% acrylamide solution; stacking gel contained 4% acrylamide solution. Samples were applied to the gel in sample buffer consisting of pH 6.7, 50 mM Tris-HCl:glycerol:water:bromphenol blue, 1:1:1:250 (v/v/v/w) expressed as ml and μg. The gels were removed from the plates, fixed and stained as above. The molecular weights were determined by the procedure described in the manufacturer technical bulletin (Ferguson, 1964; Hendrick and Smith, 1968). This consists of determining the relative mobility (Rf) of the protein in each gel relative to the tracking dye. Hundred times logarithm of (Rf x 100) is plotted against the percent gel concentration for each protein. The slope of such a plot is the retardation coefficient (K^r). From these plots individual Krs are determined for each protein and the logarithm of the negative slope is plotted against the logarithm of the molecular weight of each protein. This graph produces a linear plot from which the molecular weight of exo-ß-galactofuranosidase was determined.

Isoelectric Focusing

Isoelectric focusing of exo-ß-galactofuranosidase was performed using the PhastSystem (Pharmacia). PhastGel IEF media precast homogeneous (5%T, 3%C) polyacrylamide, pH 4.0 to 6.5, was used for the determination of the isoelectric point. The procedure employed was that described in the technical literature published by the Manufacturer of the apparatus.

NH_2-Terminal Analysis

The dansylation technique of Woods and Wang (1967) as described by Gray (1972) was used for the determination of the NH_2-terminus of the enzyme and bovine serum albumin as a reference protein. To a sample volume of 20 μl containing 75 μg of protein, was added 50 μl of 1% SDS. The mixture was heated in a boiling water bath for 2-5 minutes. After cooling, 70 μl of N-ethylmorpholine was added and mixed thoroughly. Dansyl chloride was then added (105 μl; 25 mg/ml). Reaction was allowed to proceed for 1 hour at room temperature. The labelled protein was precipitated by addition of 0.5 ml of acetone to the reaction mixture. The precipitate was washed once with 80% acetone, dissolved in 6 N HCl and held at 100^0C overnight in capped "reacti" vials. The hydrolysate was reduced to dryness by passing a stream of dry nitrogen over it. The residue was dissolved in 10 μl of acetone and was subjected to thin-layer chromatography.

Determination of the Kinetic Properties of Exo-ß-galactofuranosidase

Optimum pH for activity. Reaction mixture containing i) 400 μl of buffer (50 mM sodium citrate, pH 2 to 7.5 and 50 mM Tris-HCl, pH 8 to 9), ii) 50 μl of 1-O-ß-methyl-D-galactofuranoside (750 μg) and 30 μl of the purified galactofuranosidase preparation (60 ng of protein) was incubated for 90 minutes at 40^0C. The release of galactose after incubation with the enzyme was determined by the galactose oxidase.

pH stability profile. Sixty nanograms of enzyme preparation in 200 μl of buffer (50 mM sodium citrate, pH 2 to 7.5 and 50 mM Tris-HCl, pH 8 to 9) was incubated at 4-8^{0}C for 24 hours. After that period of time, the pH was adjusted to 4 with 0.1 N HCl or 0.1 N NaOH. Substrate (50 μl of 1-O-ß-methyl-D-galactofuranoside, 750 μg), was then added and the reaction mixture was then incubated for 120 minutes at 40^{0}C. Galactose released was determined by the galactose oxidase test.

Optimum temperature for activity. A volume of 400 μl of buffer (66 mM sodium acetate at pH 4.0) was brought to the indicated temperature. The purified enzyme was then added (30 μl, 0.12 μg of protein) with 50 μl (500μg) of 1-O-ß-methyl-D-galactofuranoside. The reaction was allowed to proceed for one hour. Galactose released was measured by the galactose oxidase test.

Temperature stability. Purified enzyme (30 μl, 0.12 μg of protein) was added to 400 μl of buffer (66 mM sodium acetate, pH 4.0) adjusted to the indicated temperature. The mixture was incubated for 45 minutes. After readjusting the temperature to 40^{0}C, 50 μl (500μg) of 1-O-ß-methyl-D-galactofuranoside was added and the reaction mixture incubated for one hour at 40^{0}C. Purified exo-ß-galactofuranosidase preparation (200 μl, 60 ng of protein) was incubate at -20^{0}C for 24 hours to determine the stability of the enzyme to freezing and thawing. Then, the preparation was thawed; 300 μl of

66 mM acetate buffer, pH 4.0, containing 500 μg of 1-O-ß-methyl-D-galactofuranoside was added and the reaction allowed to proceed for 60 minutes.

K_m and V_m determination. Km and Vm were determined on pP_2GM^{ii}s and $pP_{30}GM^{ii}$s, 1-O-ß-methyl-D-galactofuranoside, and galactofurano-oligosaccharides enriched in galactofuranotetraose or galactofuranohexaose. For this purpose, 820 μl of buffer (66 mM acetate, pH 4.0) containing substrate concentrations ranging from 0.025 to 11 μmoles of nonreducing terminal galactofuranosyl residues were added to 80 μl of purified enzyme preparations (0.7 to 3 μg of protein) and the reaction mixture incubated at 40^0C. Sample sizes of 50 μl were taken out at 15, 30, 60, 120, 180 and 240 minutes, and the reaction stopped by adding 550 μl of 0.1 M phosphate buffer, pH 8.0. The release of galactose was then determined by the galactose oxidase test. Km and Vm were determined from Cornish-Bowden plots.

Substrate specificity of the exo-ß-galactofuranosidase. 1-O-ß-Methyl-D-galactofuranoside, $Galf_{3-4}$ and $Galf_{5-6}$ linked ß-D-(1-->5), pP_2GM^{ii} and $pP_{30}GM^{ii}$ were used to test the specificity of the exo-ß-galactofuranosidase. The amount of galactose released was determined by the galactose oxidase test. The reaction mixtures were the same as described above.

Activities of purified exo-ß-galactofuranosidase preparation toward substrates for phospho-monoesterases, phosphodiesterases and other

glycosidases were determined using p-nitrophenyl-α-galactopyranoside, p-nitrophenyl-ß-galactopyranoside, p-nitrophenyl-N-acetyl-ß-glucosamine, p-nitrophenyl-phosphocholine, p-nitrophenyl phosphate and bis(p-nitrophenyl) phosphate. 200 μl of substrate (6 μmole) was added to 300 μl of buffer (66 mM acetate pH 4.0) containing 1.2 μg of enzyme preparation. The reaction was allowed to proceed at 40^0C for 150 minutes and then stopped by addition of 1 ml of 0.2 N NaOH. The amount of product released was compared to that of 1-O-ß-methyl-D-galactofuranosidase in the same conditions (galactose released was measured by galactose oxidase test).

RESULTS

Enzyme Purification

Rietschel-Berst et al. (1977) have reported the purification of extracellular exo-ß-galactofuranosidase to a nearly homogeneous state in one step using a peptidophospho-galactomannan affinity column. Discontinuous gel electrophoresis of a sample eluted from the affinity column with sodium acetate buffer, pH 4.0, showed three major protein bands; while a sample eluted with 0.1 M phosphate buffer, pH 7.0, showed two major protein bands.

Pletcher et al. (1979) also failed to purify galactofuranosidase; at least one of the contaminants was an endogeneous protease which inactivates galactofuranosidase. She attempted to circumvent this problem by the addition of phenylmethylsulfonylfluoride (PMSF) to inactivate serine proteases. However, this treatment only partially inhibited proteases in the culture filtrates.

Another protocol for protein purification was developed. Medium, from 18-20-day old cultures to which PMSF was added at a final concentration of 10^{-4} M (3.4 mg/200 ml flask) 24 hours before harvest, was filtered and concentrated in an Amicon ultrafiltration apparatus (MW cut off 30,000). There was no detectable galactofuranosidase activity in the filtrate, all the activity remaining in the retentate.

This indicates that galactofuranosidase MW is $>$ 30,000.

Anion Exchange Chromatography I

Aliquots from Amicon-concentrated 18-21 day culture filtrates were subjected to ion exchange chromatography on a DEAE-cellulose column in 50 mM sodium acetate buffer, pH 5.0. The distribution of protein and galactofuranosidase activity is shown in Figure 1. The fractions collected from the column were measured at 280 nm and assayed for exo-ß-galactofuranosidase activity. The bulk of the protein (60%) did not bind to the column and it is in this effluent that exo-ß-galactofuranosidase activity was found. Total recovery of the exo-ß-galactofuranosidase activity from this first anion exchange column was 74%. Even though exo-ß-galactofuranosidase does not bind to the column at pH 5.0, 40% of the total protein bound and was removed. Based on the increase in specific activity, this step represents about a 1.2-fold purification of exo-ß-galactofuranosidase from the culture filtrate. On that basis, it was determined that anion exchange chromatography at pH 5.0 was a good first step to resolve exo-ß-galactofuranosidase activity from other proteins present in the culture filtrate that may interfere in later steps.

Anion Exchange Chromatography II

Exo-ß-galactofuranosidase activity recovered from the first anion exchange was applied to a second anion exchange column (DEAE-cellulose) previouly equilibrated with 50 mM MOPS buffer, pH 7.5 (Figure 2).

Figure 1. Elution Profile of Exo-ß-Galactofuranosidase from DE-52 Anion Exchange Column I

Culture filtrates were concentrated and applied to a DEAE-cellulose column (3 x 28 cm) as described in Materials and Methods. The column was pre-equilibrated with 50 mM sodium citrate, pH 5.0 and washed with the same buffer. The protein was eluted with a stepwise gradient of NaCl. Fractions of 7.5 ml were collected. Protein, (___), was monitored by absorbance at 280 nm. Exo-ß-galactofuranosidase activity, (-----), was determined on 200 μl samples of every other fraction; galactose released was estimated from the increase in absorbance at 410 nm resulting from the coupled oxidation of galactose and o-cresol by galactose oxidase and peroxidase, respectively. Column fractions containing galactofuranosidase activity as indicated by the bar above the galactofuranosidase peak of activity were pooled.

On this figure, as well as on all following figures, exo-ß-galactofuranosidase activity (----) represents a curve drawn by a computer program using all the gathered data.

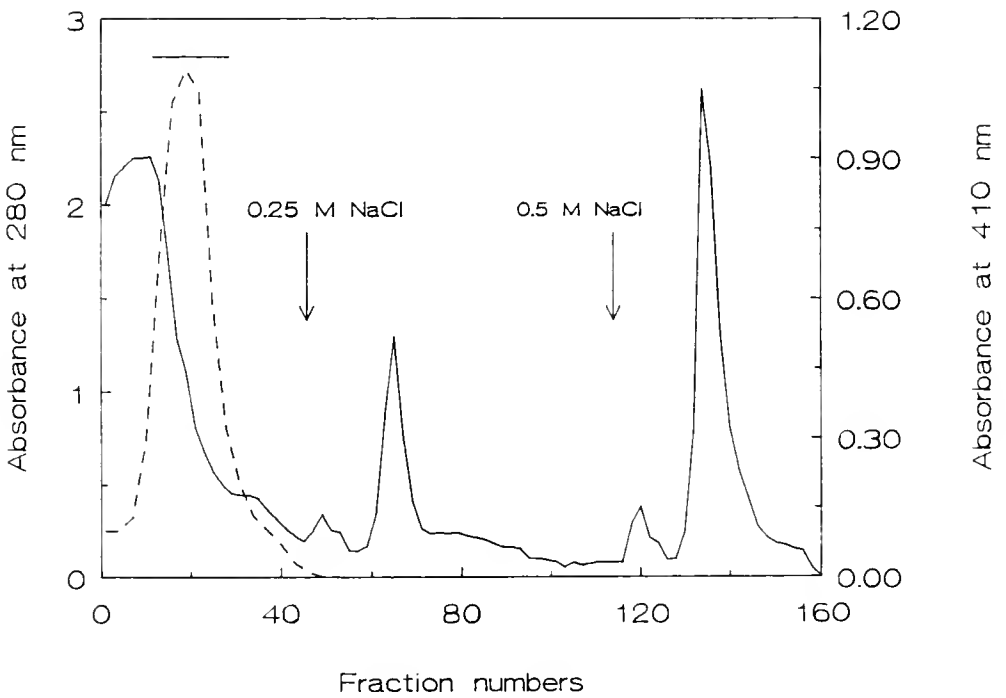
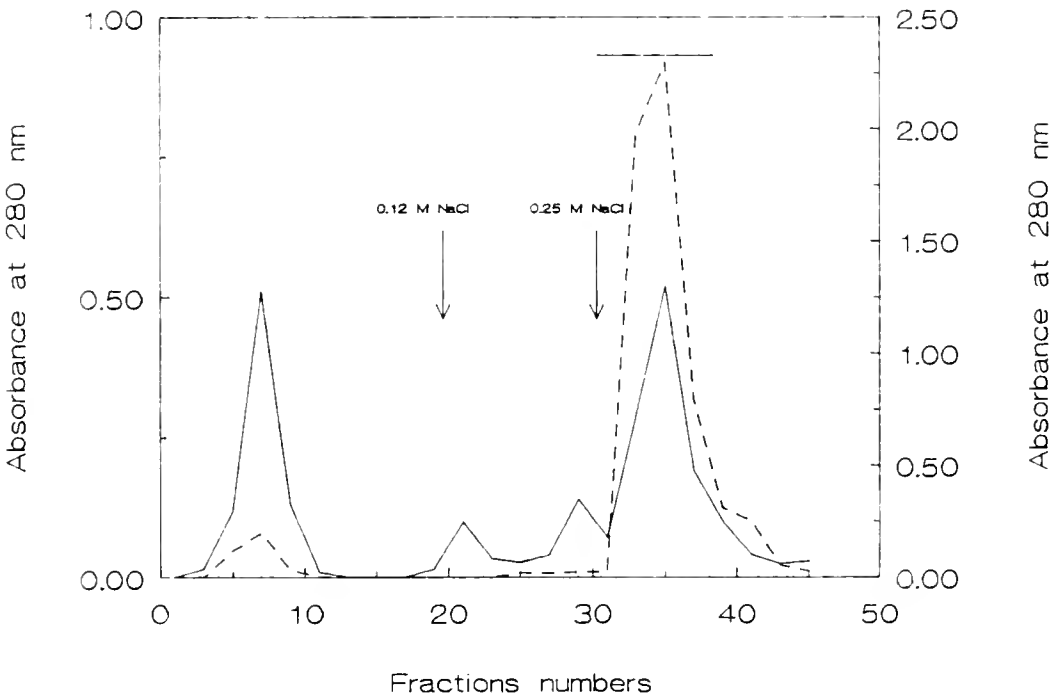

Absorbance at 280 nm
Absorbance at 410 nm
0.25 M NaCl
0.5 M NaCl
Fraction numbers
0
40
80
120
160
0
1
2
3
0.00
0.30
0.60
0.90
1.20

Figure 2. Fractionation of Partially Purified Exo-ß-Galactofuranosidase on DE-52 Anion Exchange Column II

Galactofuranosidase preparation (10 ml, 7 units) obtained from DE-52 Anion Exchange Column I, was applied to a second DEAE cellulose column previously equilibrated with 50 mM MOPS buffer, pH 7.5. Fractions size of 2.5 ml were collected. Protein was eluted with a stepwise gradient of NaCl. Protein, (___), was monitored by absorbance at 280 nm. Galactofuranosidase activity, (----), was determined on 100 μl samples of every other fraction; galactose released was estimated by an increase in absorbance at 600 nm resulting from the increase in reducing sugars assayed by Nelson's test for reducing sugars. Fractions containing galactofuranosidase activity as indicated by the bar were pooled.


1.00
0.50
0.00
2.50
2.00
1.50
1.00
0.50
0.00
0.12 M NaCl
0.25 M NaCl
0
10
20
30
40
50
Absorbance at 280 nm
Absorbance at 280 nm
Fractions numbers

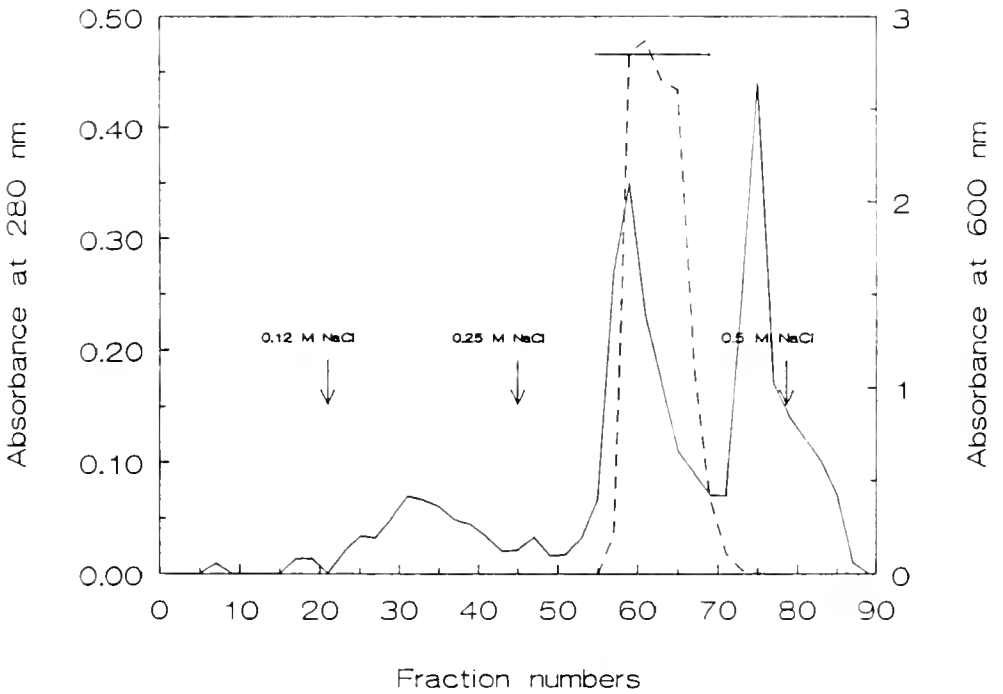
Protein eluted in two major and two minor peaks. Half of the protein, but almost none of the exo-ß-galactofuranosidase activity came through in the effluent (first peak). The two minor protein peaks have no galactofuranosidase activity. Exo-ß-galactofuranosidase activity is eluted in the last protein peak with 25 mM sodium citrate containing 0.25 M NaCl. Protein peaks without galactofuranosidase activity were discarded without further analysis. Approximately 73% of the galactofuranosidase activity applied to the column was recovered in that protein peak.

Cation Exchange Chromatography

The protein fraction containing galactofuranosidase activity obtained from DEAE-cellulose chromatography II was applied to a CM-sepharose column in 12 mM sodium tartrate buffer, pH 3.0 (Figure 3). None of the protein came through in the effluent. One minor peak of protein without exo-ß-galactofuranosidase activity was eluted with 0.12 M NaCl. Galactofuranosidase activity, which was found as a shoulder on the protein peak, eluted with 25 mM sodium citrate containing 0.25 M NaCl, pH 5.0. A second peak of protein eluted with the same salt concentration but did not have any galactofuranosidase activity.

Figure 3. Fractionation of the Galactofuranosidase Preparation Obtained from DEAE-Cellulose Chromatography on CM-Sepharose by Cation Exchange

Fractions containing galactofuranosidase activity (10 units) obtained from DEAE-cellulose column II as described in "Materials and Methods", was loaded onto a CM-sepharose column (2 x 25 cm) pre-equilibrated with 12.5 mM sodium tartrate buffer, pH 3.0. Protein was eluted with a stepwise gradient of NaCl. Fractions of 2ml were collected. Protein, (___), was monitored by absorbance at 280 nm. Galactofuranosidase activity, (----), was determined on 100 μl samples of every other fraction; galactose released was estimated by an increase in absorbance at 600 nm resulting from the increase in reducing sugars assayed by Nelson's test for reducing sugars. Fractions with galactofuranosidase activity, as indicated by the bar, were pooled.


0.50
0.40
0.30
0.20
0.10
0.00
Absorbance at 280 nm
3
2
1
0
Absorbance at 600 nm
0.12 M NaCl
0.25 M NaCl
0.5 M NaCl
0
10
20
30
40
50
60
70
80
90
Fraction numbers

Superose-12 Gel Filtration I

Fractions containing exo-ß-galactofuranosidase activity from CM-sepharose chromatography was loaded onto a gel filtration column (Superose 12) equilibrated with 10 mM sodium acetate buffer, pH 4.0 containing 10 mM NaCl (Figure 4). Two distinct peaks of protein eluted as the column was washed with the same buffer. Galactofuranosidase activity was associated with the first peak of protein. Fractions with exo-ß-galactofuranosidase activity were then concentrated as indicated in "Materials and Methods" and reapplied to the same column.

Superose 12 Gel Filtration II

Proteins with galactofuranosidase activity recovered from several gel filtration I (Superose 12) steps were pooled, concentrated and reapplied to the same gel filtration column (Figure 5). Protein eluted as a single peak which contained exo-galactofuranosidase activity.

Homogeneity of the Purified Exo-ß-Galactofuranosidase

The protein content of intermediate and final exo-ß-galactofuranosidase preparations were determined by BCA protein assay (Pierce). For comparative purposes, samples from each step and with various protein concentrations were assayed for exo-ß-galactofuranosidase activity using 1-O-ß-methyl-D-galacto-furanoside as substrate.

Figure 4. Elution Profile of Exo-ß-D-Galactofuranosidase Activity on Superose-12 FPLC Gel Filtration Column I

A galactofuranosidase preparation (200μl), obtained from CM-sepharose cation exchange column and concentrated as described in the Materials and Methods, was applied to a Superose-12 FPLC gel filtration column (1.5 x 31 cm) previously equilibrated with 10 mM acetate buffer, pH 4.0, containing 10 mM NaCl. Protein elution was done with the same buffer at a flow rate of 0.12 ml/minute. Fractions of 250 μl were collected. Protein, (___), was monitored by absorbance at 280 nm. Galactofuranosidase activity, (----), was determined on 20 μl samples of every other fraction; galactose released was estimated by an increase at 600 nm resulting from the increase of reducing sugars as assayed by Nelson's test for reducing sugars.

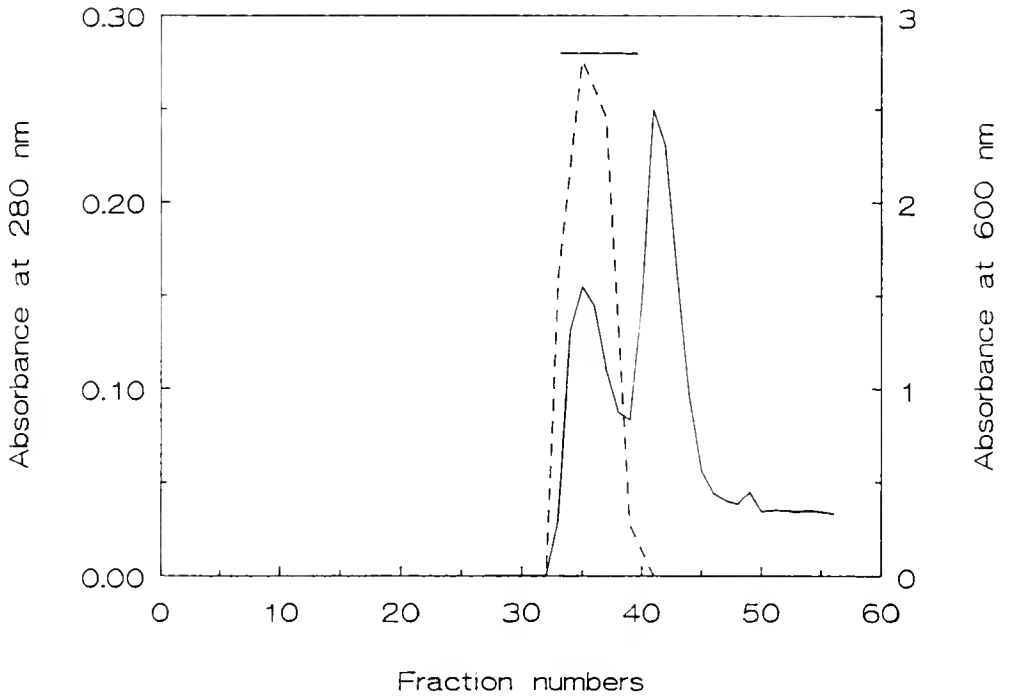
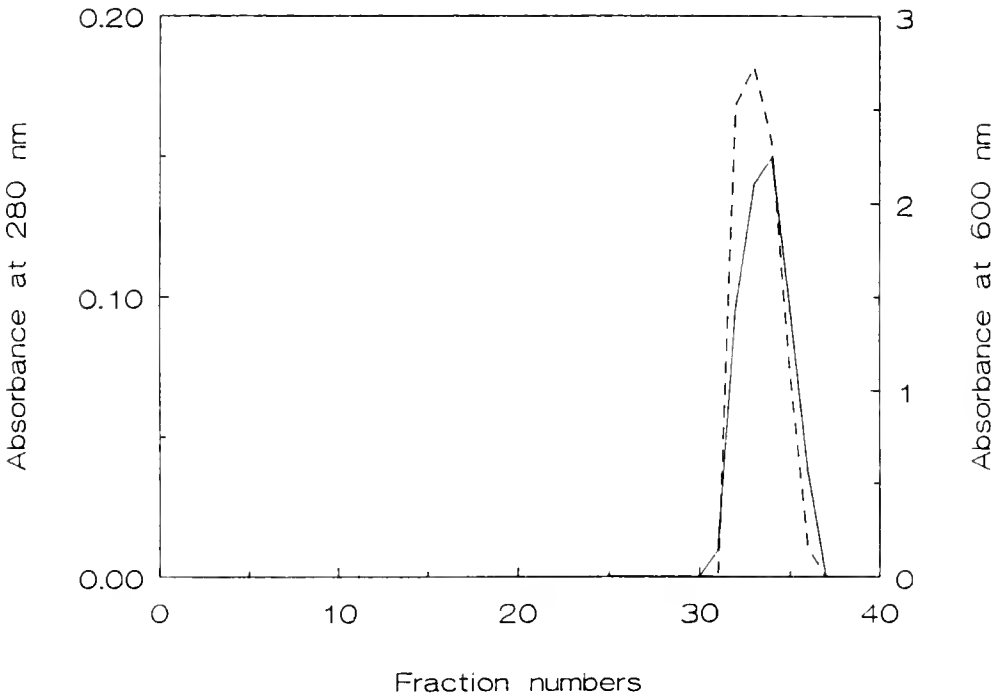

0.30
0.20
0.10
0.00
Absorbance at 280 nm
3
2
1
0
Absorbance at 600 nm
0
10
20
30
40
50
60
Fraction numbers

Figure 5. Second Gel Filtration of Exo-ß-Galactofuranosidase Activity on Superose-12 FPLC Column

Galactofuranosidase positive fractions from the first gel filtration were collected, concentrated 10-fold, filtered through a 0.2 MSI filter apparatus and 200 μl loaded onto the same Superose-12 FPLC gel filtration column. Fractions of 250 μl were collected. Protein, (___), was monitored by the absorbance at 280 nm. Galactofuranosidase activity, (----), was assayed on 20 μl samples of every other fraction and galactose released was estimated as described in figure 4.


0.20
0.10
0.00
Absorbance at 280 nm
3
2
1
0
Absorbance at 600 nm
0
10
20
30
40
Fraction numbers

The activity of exo-ß-galactofuranosidase and total protein present at each step are shown in Table 1. The crude preparation contained 20 units of activity and 77 mg of protein per 1 L of medium. The 5-step procedure resulted in a 100-fold purification of exo-ß-galactofuranosidase with a 45% recovery of activity. Thus, approximately 1% of the protein in the crude preparation was exo-ß-galactofuranosidase. Aliquots of exo-ß-galactofuranosidase from gel filtration chromatography I and/or II were analyzed by SDS-PAGE, by non-denaturating PAGE (not shown) and by IEF.

SDS_PAGE. Aliquots of gel filtration I and II were analyzed by SDS-PAGE (Figure 6). Gels, stained with 0.1% Coomassie Brillant Blue R-250 following Bio-Rad's silver staining procedure, showed a major band with an approximate molecular weight of 75 kd in lane 2 corresponding to gel filtration I. Lane 3 represents protein after gel filtration II.

The above results suggest that exo-ß-galactofuranosidase has an apparent molecular weight of 75 kd. Molecular weight was calculated from a calibration curve plotted on a semi-log paper of the molecular weight vs relative mobility in SDS-PAGE of low molecular weight protein standards (Lanes 1 and 5).

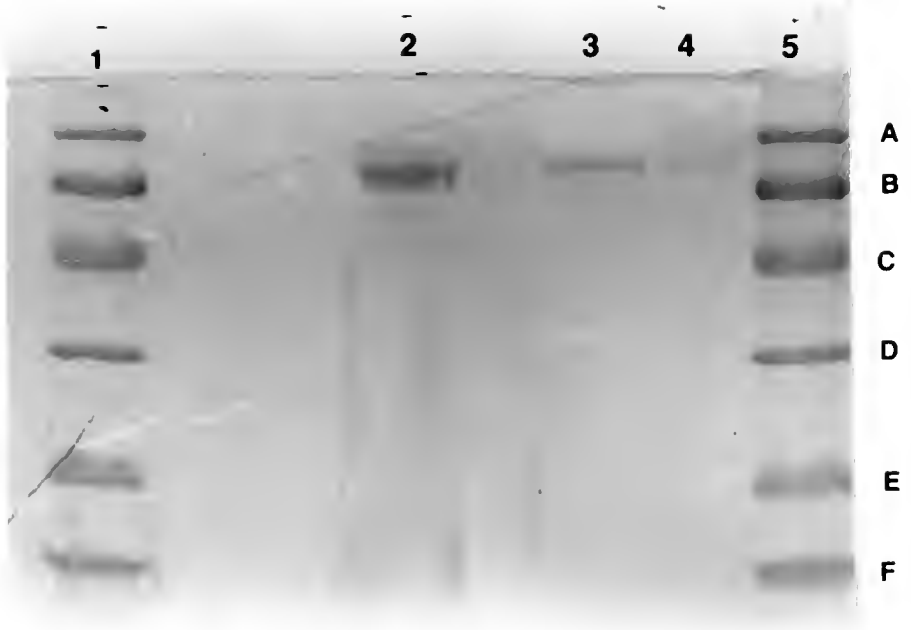
Table 1. Purification of Exo-ß-galactofuranosidase

Step	protein mg	activity Units	Specific act. Units/mg	Recovery %	fold purification
Crude	76.8	19.2	0.25	100	-
DEAE-I	47.6	14.3	0.3	74	1.2
DEAE-II	13.0	10.4	0.8	54	3.2
CM-Sepharose	1.2	5.8	4.8	30	19.2
FPLC-II	0.3	8.6	25.2	45	100

Figure 6. Photograph of Exo-ß-Galactofuranosidase Following SDS-PAGE

Samples were diluted 1:5 (v/v) in SDS sample buffer and loaded into wells of gels cast in 7 x 10 cm glass plates (0.5 mm). Electrophoresis was carried out at constant current of 8 mA until the dye front reached the bottom of the gel (30-45 min) at a constant current of 8 mA. Following electrophoresis, the gels were stained with Coomassie Brillant Blue R-250 in 40% methanol, 10% acetic acid. . Both molecular weight standard and samples were heated at 95^0C for 5 minutes.

The standard mixture contained (a) phosphorylase b (97.4 kd), (b) bovine serum albumin (66.2 kd), (c) ovalbumin (45.0 kd), (d) carbonic anhydrase (31.0 kd), (e) soybean trypsin inhibitor (21.5 kd), (f) lysozyme (14.0kd).
Lanes 1 & 5 .....Molecular weight standard mixture as shown above
Lane 2.....Exo-ß-galactofuranosidase preparation after first gel filtration
Lane 3.....Exo-ß-galactofuranosidase preparation after second gel filtration
The band in Lane 4 represents a spillover from Lane 3.


1
2
3
4
5
A
B
C
D
E
F

Non-Denaturating PAGE. Exo-ß-galactofuranosidase preparations from gel filtration II were subjected to non-denaturating PAGE using gels with different polyacrylamide concentrations (7, 8, 9 and 10%). After staining with 0.1% Coomassie Brilliant Blue R-250 following Bio-Rad silver staining procedure, two bands were detected. Proteins in these bands had an approximate molecular weight of 150 kd and 70 kd. The molecular weights were determined by the procedure described in the Sigma technical bulletin No. MKR-137 as discused in "Material and Methods".

Isoelectric Focusing (IEF). Exo-ß-galactofuranosidase preparations from gel filtration II were isoelectrically focused on Phast Gel IEF (4-6.5 pI) medium (Pharmacia). After staining the gel with Bio-Rad silver stain, a single band (Figure 7) with a pI of 4.35 was detected in the lane containing enzyme fraction from gel filtration II (Lane 2). The pI of exo-ß-galactofuranosidase was calculated from a calibration curve plotted as a function of pI calibration standards vs distance from the cathode (Lanes 1 and 3). Isoelectric focusing followed by slicing of the gel and assaying for galactofuranosidase activity in the sliced bands revealed one major band with activity around pH 4.0 as shown of Figure 8.

Figure 7. Photograph of Exo-ß-Galactofuranosidase Following IEF

Isoelectric focusing of galactofuranosidase preparation was performed using the PhastSystem (Pharmacia). PhastGel IEF media precast polyacrylamide (5%T, 3%C), pH 4.0 to 6.5 was used. Following IEF, gels were stained with silver stain.
pI markers were (a) glucose oxidase (pI-4.15), (b) soybean trypsin inhibitor (pI-4.55), (c) ß-lactalbumin (pI-5.20), (d) bovine carbonic anhydrase (pI-5.85)
Lanes 1 & 3.... pI calibration mixture
Lane 2 ..... Exo-ß-galactofuranosidase preparation, containing 60 ng of protein.

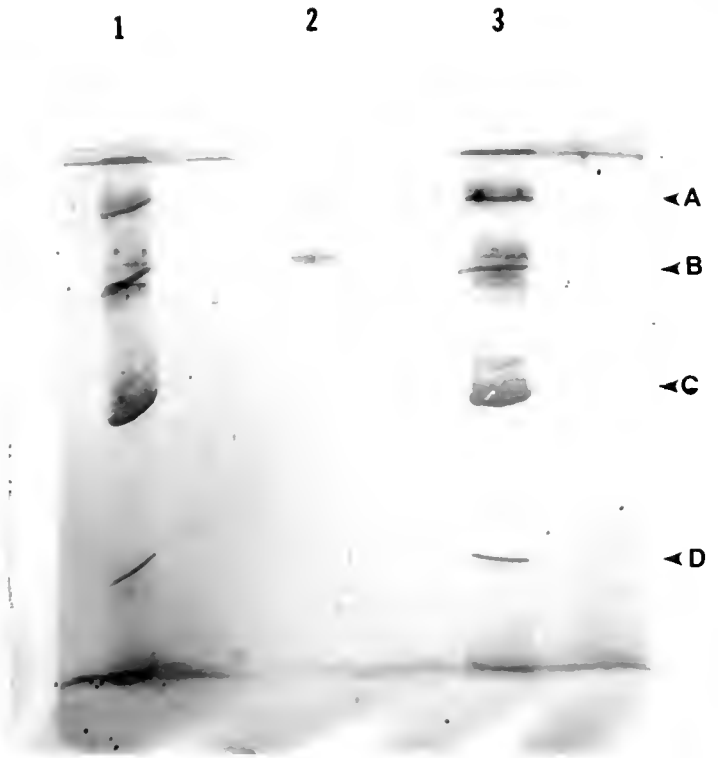
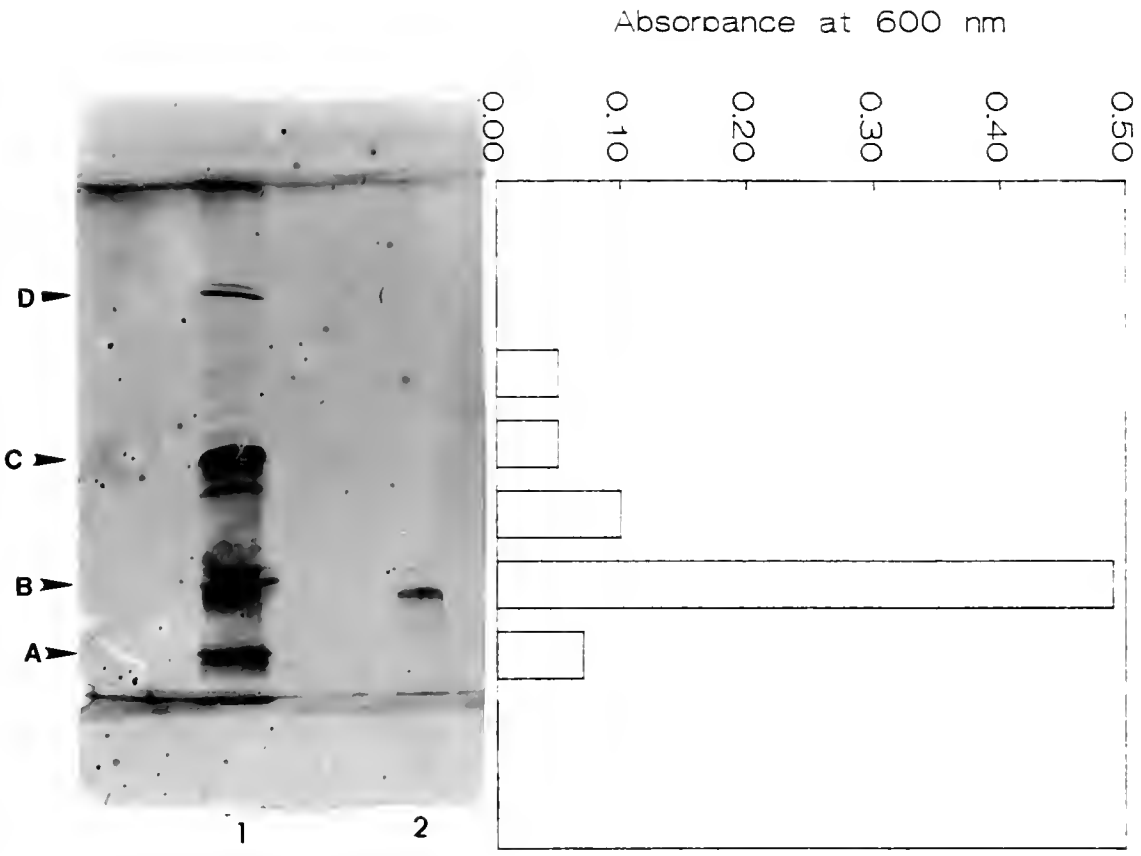

1
2
3
A
B
C
D

Figure 8. Zymogram of Exo-ß-Galactofuranosidase Following IEF

Isoelectric focusing of galactofuranosidase preparation was performed using the PhastSystem (Pharmacia). PhastGel IEF media precast polyacrylamide (5%T, 3%C), pH 4.0 to 6.5 was used. Following IEF, gels were stained according to manufacturer protocol (Bio-Rad) with silver stain.
pI markers were (a) glucose oxidase (pI-4.15), (b) soybean trypsin inhibitor (pI-4.55), (c) ß-lactalbumin (pI-5.20), (d) bovine carbonic anhydrase (pI-5.85)
Lane 1..... pI calibration mixture
Lane 2 ..... Exo-ß-galactofuranosidase preparation, containing 60 ng of protein.

To test for exo-ß-galactofuranosidase activity, half of the gel was cut out and incubated in 66 mM acetate buffer and the other half was stained with silver stain. Bands of the unstained half were then sliced out as indicated. Gel pieces were passed through a 5 cc syringe and made up to 1 ml in 66 mM sodium acetate buffer pH 4.0. Three hundred microliters of that solution were added to 200 μl of susbtrate (1-O-ß-D-methyl galactofuranoside, 500 μg). The reaction was allowed to proceed for 20 hours at 40^0 C. Galactose released was measured by the Nelson's test for reducing sugars at 600 nm.


Absorbance at 600 nm
0.00
0.10
0.20
0.30
0.40
0.50
D
C
B
A
1
2

Carbohydrate Content

Carbohydrate content of exo-ß-galactofuranosidase was determined on 16 μg of protein in 100 μl of 10 mM, pH 4.0 sodium acetate buffer. The phenol-sulfuric acid assay was used. The results showed that the reaction contained 2.7 μg (15% carbohydrate) by weight.

A sample containing 50 μg of protein was treated with 2 N HCl for 3 hours at 100° C. The reaction mixture was analyzed by paper chromatography and showed to contain mannose; no galactose was detected (data not shown).

N-Terminal Amino Acid Analysis

A sample containing about 2 μg of galactofuranosidase did not react with DANSYL.Cl; a control reaction with bovine serum albumin reacted. This suggests that exo-ß-galactofuranosidase amino-terminal group is blocked.

Properties of Exo-ß-Galactofuranosidase

pH Activity Profile

When assayed over the pH values 2 to 9, exo-ß-galactofuranosidase appeared to be active over a defined pH range (3.5 to 6.0) (Figure 9A). The optimal pH for exo-ß-galactofuranosidase activity was at pH 4.0-4.5. Less than 50% of the activity was observed at pH 6 and above.

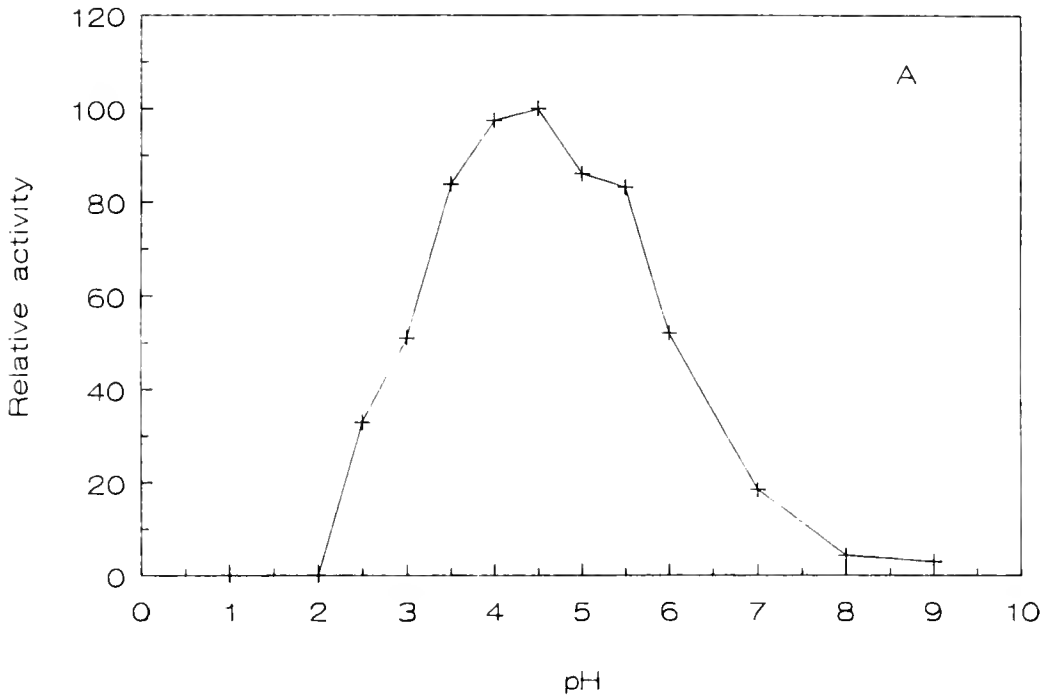
pH Stability

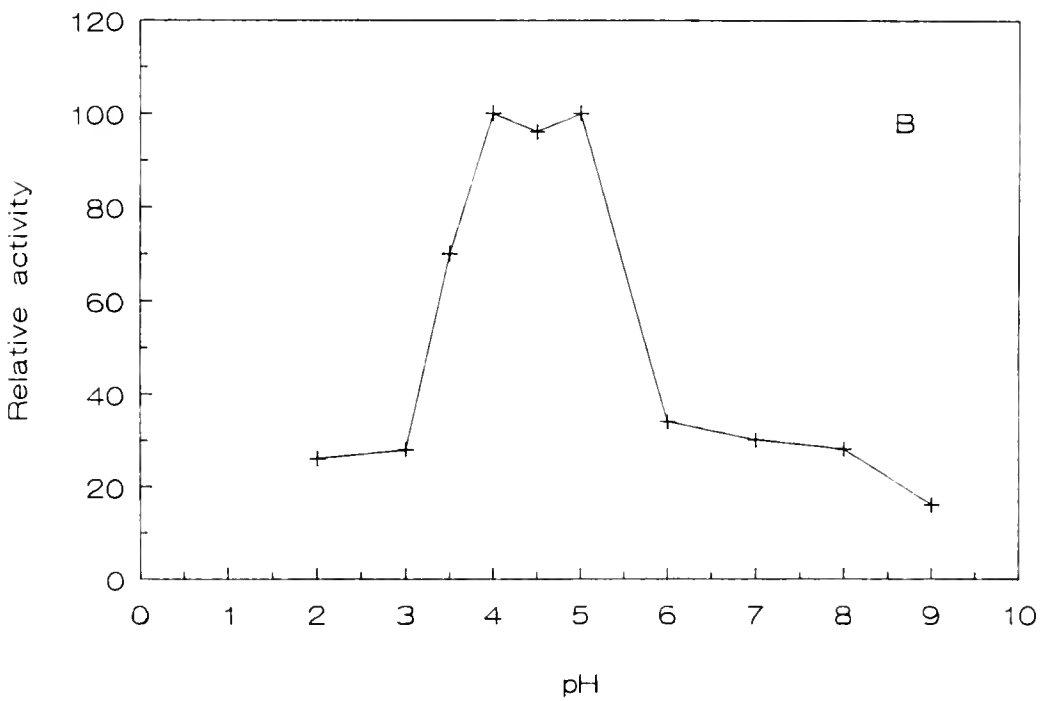
After incubating equal aliquots of enzyme in buffers of various pH

Figure 9. Optimum pH for Activity and pH Stability of Exo-ß-galactofuranosidase

Panel A: optimum pH for activity. Reaction mixture containing i) 400 μl of buffer (50 mM sodium citrate, pH 2 to 7.5 or 50 mM Tris-HCl, pH 8 to 9), ii) 50 μl of 1-O-ß-methyl-D-galactofuranoside (750 μg) and iii) 30 μl of purified exo-ß-galactofuranosidase preparation (60 ng of protein) was incubated at 40^0C for 90 minutes. Galactose released was measured by the increase in absorbance at 410 nm following the coupled oxidation of galactose and o-cresol by galactose oxidase and peroxidase, respectively. Galactose released (0.138 μmoles), at pH 4.5, was given the relative value of 100% activity.

Panel B: pH stability profile. Sixty nanograms of enzyme preparation in 400 μl of buffer (50 mM sodium citrate, pH 2 to 7.5 or 50 mM Tris HCl, pH 8 to 9) was incubated at 4^0C for 24 hours. After that period of time, the pH was adjusted to 4 with 0.1 N HCl or 0.1 N NaOH. Substrate, 50 μl of 1-O-ß-methyl-D-galactofuranoside (750 μg) was then added. The reaction mixture was incubated at 40^0C for 120 minutes. Galactose released was measured by the increase at 410 nm following the coupled oxidation of galactose and o-cresol by galactose oxidase and peroxidase, respectively. Galactose released (0.158 μmoles0, at pH 4.0, was given the relative value of 100% activity.


A
Relative activity
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10
pH


B
Relative activity
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10
pH

values at room temperature for 24 hours, the residual galactofuranosidase activity in the preparations was determined at pH 4.0. As shown in Figure 9B, exo-ß-galactofuranosidase is stable over a narrow pH range (3.5 to 5.5) in the conditions used. The enzyme was most stable at pH 4-5 which is also the optimal pH for activity of exo-ß-galactofuranosidase.

Temperature Optimum

The optimal temperature for exo-ß-galactofuranosidase activity was determined by assaying purified enzyme preparations over a wide range of temperatures as described in "Materials and Methods". As shown in Figure 10A the optimal temperature for exo-ß-galactofuranosidase activity was 40^0C. The rapid decline of activity after 60^0C is probably due to protein denaturation.

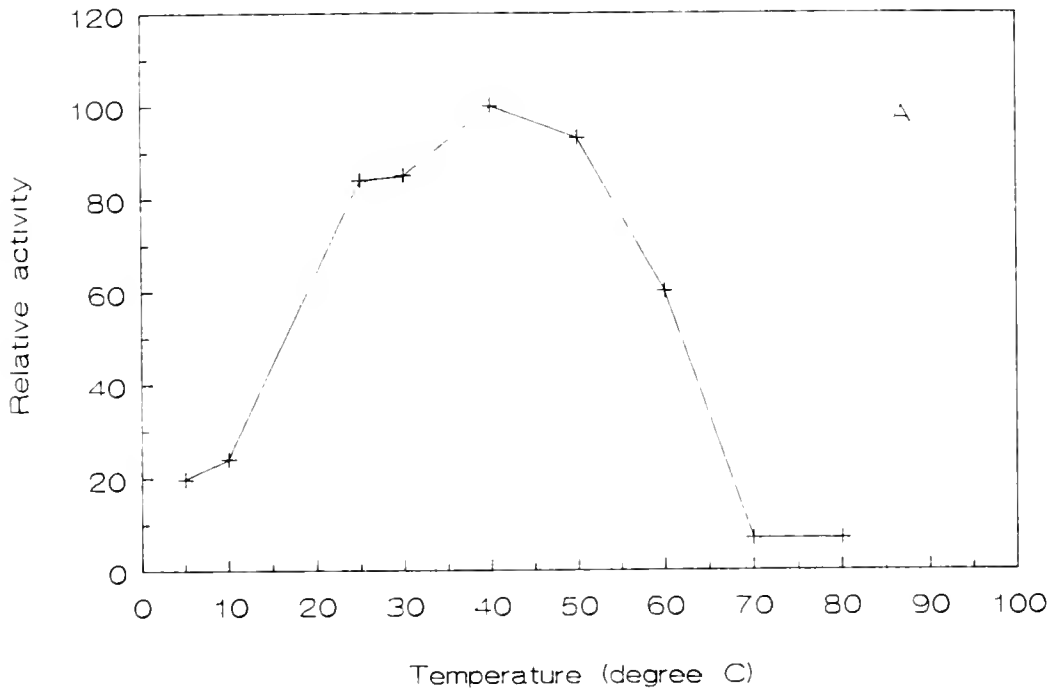
Temperature Stability

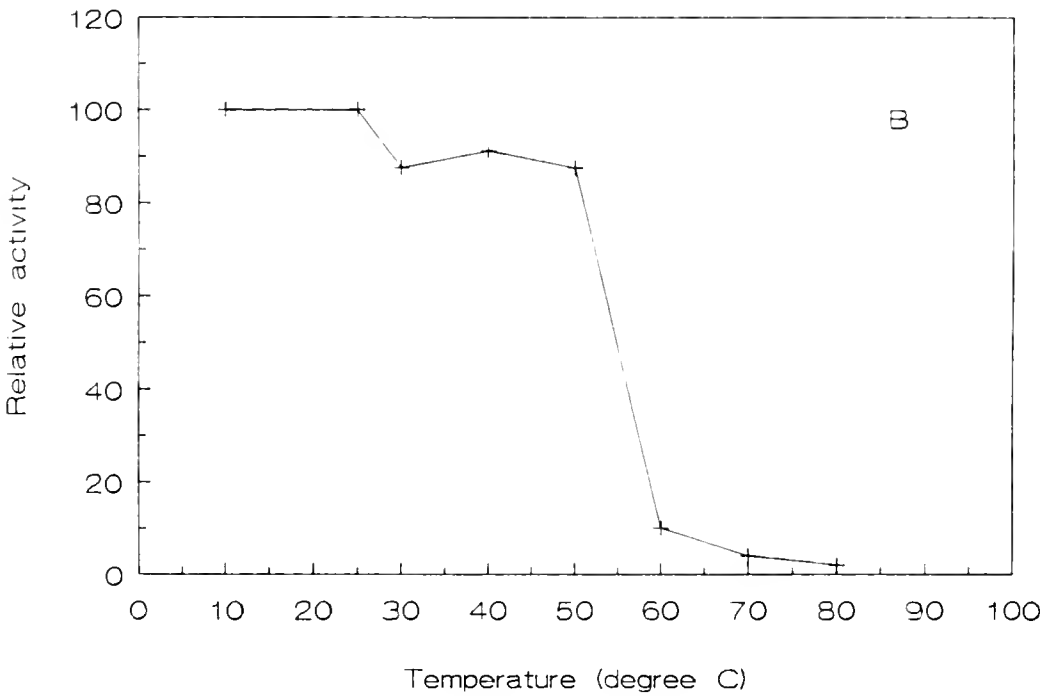
The thermal stability of exo-ß-galactofuranosidase determined following 45 minutes incubation at indicated temperatures (Figure 10B) suggests that exo-ß-galactofuranosidase was stable up to 45 minutes between 4^0C to 50^0C. The enzyme looses nearly all of its activity at 60°C. About 94% of the enzyme activity was recovered following freezing and thawing of the enzyme preparation. The loss of enzyme activity at 60° C in the presence of substrate (top panel) compared with that in the absence of substrate (lower panel) showed decreases of 40% and 90%, respectively. This suggests that substrate

Figure 10. Optimum Temperature and Temperature Stability for Activity of Exo-ß-galactofuranosidase

Panel A: Optimum temperature for activity. Four hundred microliters of buffer (66 mM acetate at pH 4.0) was brought to the indicated temperature. The enzyme was then added (30 μl, 0.12 μg of protein) with 50 μl (500 μg) of 1-O-ß-methyl-D-galactofuranoside. The reaction was allowed to proceed for 60 minutes. Galactose released was measured by the increase at 410 nm following the coupled oxidation of galactose and o-cresol by galactose oxidase and peroxidase respectively. The release of 0.146 μmoles of galactose at 40^0C was given the relative value of 100%.

Panel B: Temperature stability. Thirty microliters of enzyme preparation (0.12 μg of protein) was added to 400 μl of buffer (66 mM acetate, pH 4.0) adjusted to the indicated temperature. The reaction mixture was incubated for 45 minutes. After readjusting the temperature to 40^0C, 50 μl (500 μg) of 1-O-ß-methyl-D-galacto-furanoside was added and the reaction mixture incubated for one hour at 40^0C. Activity at 8^0C in which 0.135 μmoles of galactose were released was given the relative value of 100%.


120
100
80
60
40
20
0
Relative activity
A
0 10 20 30 40 50 60 70 80 90 100
Temperature (degree C)


120
100
80
60
40
20
0
Relative activity
B
0 10 20 30 40 50 60 70 80 90 100
Temperature (degree C)

provides protection from thermal inactivation.

Products Derived from pP_2GM and $pP_{30}GM$ Incubated with exo-ß-Galactofuranosidase

Exo-ß-galactofuranosidase generates only one type of product after limited or extensive hydrolysis of pPGMs. As depicted in Figures 11 and 12, the only product formed from the action of exo-ß-galactofuranosidase on pPGMs and detected by paper chromatography and gel filtration on Bio-Gel P4 is galactose. The data in Figure 11 confirmed that the enzyme is an exo-acting enzyme which sequentially catalyzes the removal of ß-(1-->5)-galacto-furanoside from the nonreducing terminal end of the galactofuran chains.

Peptidophosphogalactomannan ($pP_{30}GM$), with an observed optical rotation of -0.214 (10.70 μmoles of galactofuranoside/ml), was treated with purified exo-galactofuranosidase. Galactose released by the action of the enzyme was detected as a change in the observed optical rotation (Figure 13A).

The activity of exo-ß-galactofuranosidase resulted in a change of 0.1 degree (3.2 μmoles of galactose/ml) over a period of 110 hours. This indicated that 29.7% of total galactose has been released.

Peptidophosphogalactomannan (pP_2GM) was incubated with purified exo-ß-galactofuranosidase and the product formed was detected as a change in the observed rotation (Figure 13B). Starting with an observed optical rotation of -0.445 degree (22.75 μmoles of galactofuranoside/ml) for pP_2GM^{ii}, release

Figure 11. Photograph of Paper Chromatograms of pPGMs after Treatment with Exo-ß-galactofuranosidase.

Sample of $pPGM^{ii}$s (10 mg) was treated with exo-ß-galactofuranosidase as described under "Materials and Methods". Samples were taken out after 15 and 30 minutes and spotted on Whatman No. 3 paper. Development of chromatogram was carried out for 20 hours using solvent consisting of n-butanol:pyridine:water, (6:4:3), (v/v/v). Reducing sugars were detected on paper chromatogram with alkaline siver nitrate.

Lanes 1 & 2.....$pP_{30}GM^{ii}$ after 15 and 30 minutes, respectively

Lane 3.....Galactose (Gal) and Mannose (Man)

Lanes 4 & 5.....pP_2GM^{ii} after 15 and 30 minutes respectively


Man
Gal
1
2
3
4
5

Figure 12. Gel Filtration of Galactofuranosidase Treated-$pP_{30}GM^{ii}$

A reaction mixture containing i) 7 μmoles of non-reducing terminal galactofuranosyl residues in $pP_{30}GM^{ii}$, ii) 40 μl of enzyme preparation (1.5 μg of protein) and iii) 500 μl of 66 mM sodium acetate buffer at pH 4.0 was incubated at 40^0C for 24 hours. At the end of that period of time, the mixture was loaded onto a Bio Rad P2 gel filtration column previously equilibrated with distilled deionized water. Elution was performed with distilled deionized water. Fractions of 2 ml were collected. Every other fraction was assayed for total carbohydrate, protein, and total phosphate. Total carbohydrate (___) was determined by the phenol-sulfuric acid method. Protein (_ _)was determined by the BCA protein assay from Pierce. Total phosphate (----) was determined by the ashing technique of Ames.

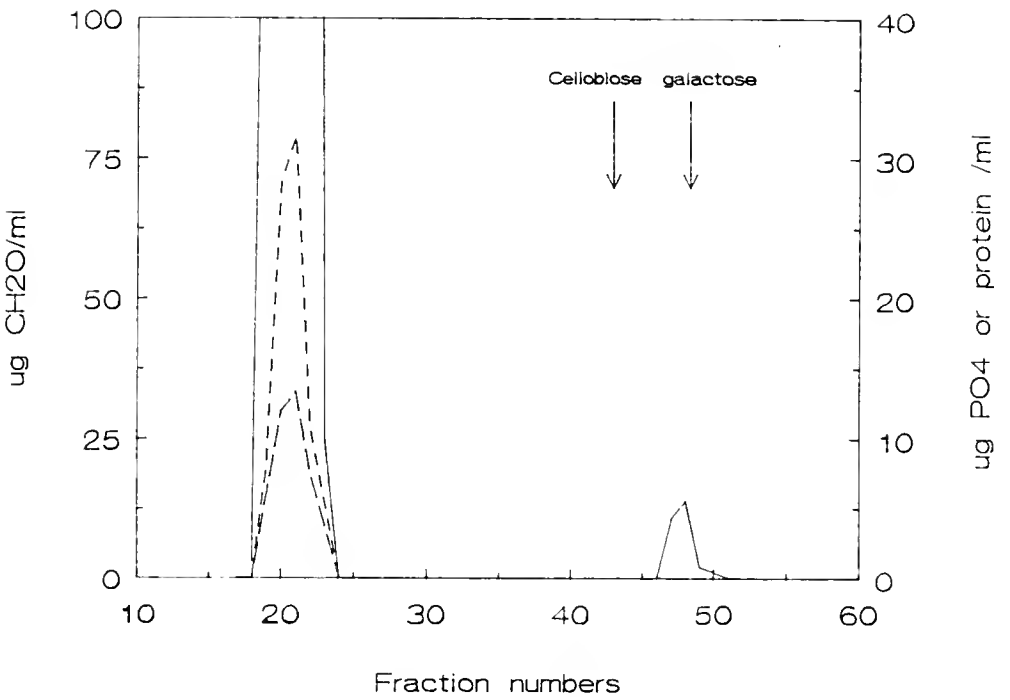
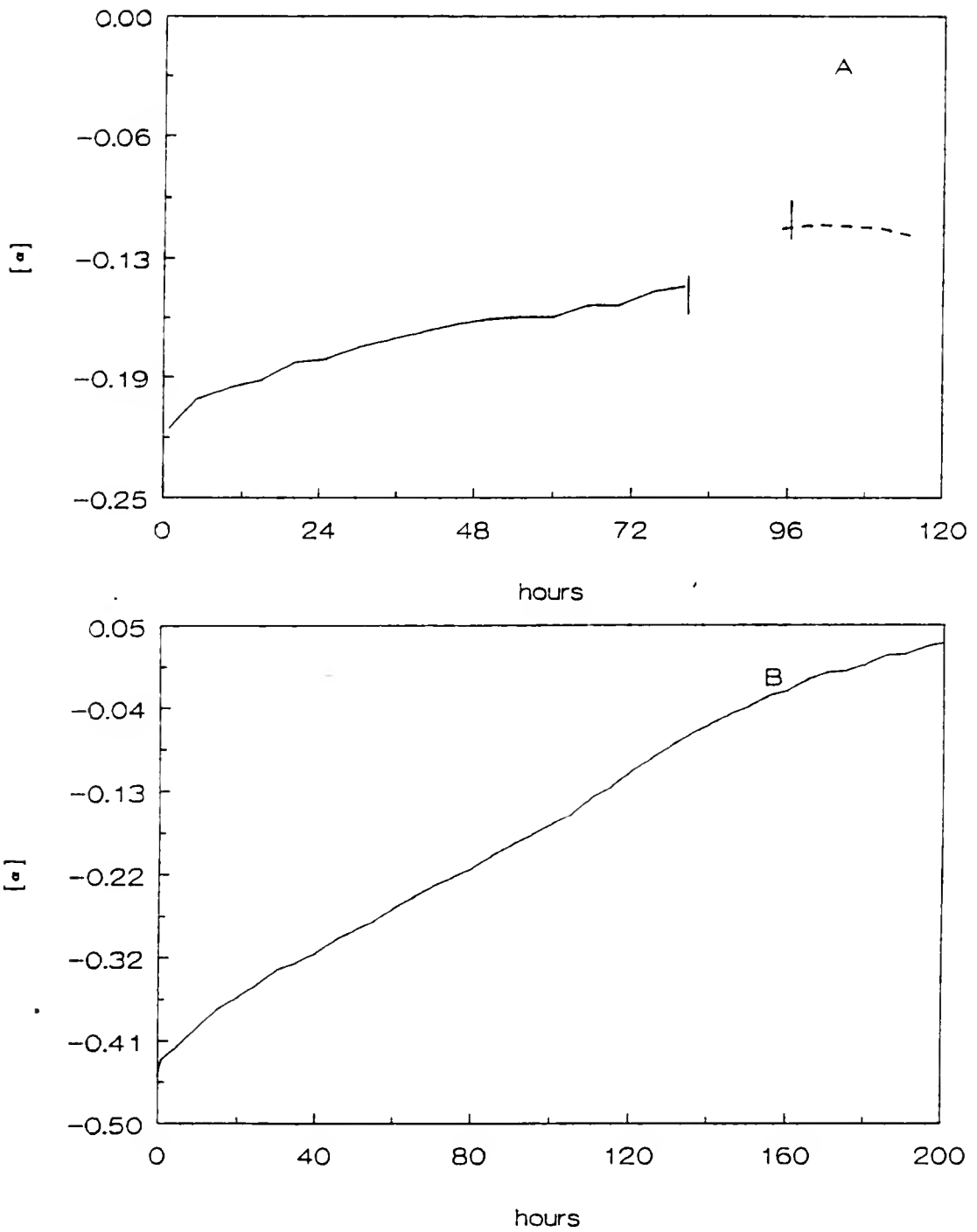

100
75
50
25
0
40
30
20
10
0
10
20
30
40
50
60
Cellobiose
galactose
ug CH2O/ml
ug PO4 or protein /ml
Fraction numbers

Figure 13. Activity of Purified Exo-ß-Galactofuranosidase on $pPGM^{ii}$s

A reaction mixture containing 2 to 4 μmoles of non-reducing terminal galactofuranosyl residues, 200 μl of enzyme preparation (6 μg protein) and 1.5 ml of 66 mM acetate buffer at pH 4.0 was incubated in a 1 dm cuvette in a Jasco DIP-360 polarimeter. Observed optical rotation was recorded every 60 minutes and the reaction was allowed to proceed for 120 or 200 hours at room temperature as indicated.

Panel A. $pP_{30}GM^{ii}$

Panel B. pP_2GM^{ii}


0.00
−0.06
−0.13
−0.19
−0.25
[α]
A
0
24
48
72
96
120
hours
0.05
−0.04
−0.13
−0.22
−0.32
−0.41
−0.50
[α]
B
0
40
80
120
160
200
hours

of galactose catalyzed by the action of exo-ß-galactofuranosidase, resulted in a change of 0.483 degree (15.33 μmoles of galactose/ml) over a period of 200 hours. These data indicate that 67.4% percent of total galactose has been released by the enzyme.

These data agree with those obtained by measuring the galactose released using the galactose oxidase assay (Table 2). Quantitative analysis of pPGMs before and after treatment with purified exo-ß-galactofuranosidase is presented in Table 3. The relatively linear relationship between the change in observed optical rotation versus time is an indication that there is negligible product inhibition. This result was confirmed by using various concentrations of galactose as inhibitor of exo-ß-galactofuranosidase catalyzed hydrolysis of 1-O-ß-methyl-D-galactofuranoside. There was little inhibition by galactose over the range of 1.38 mM to 4.16 mM (data not shown).

Km and Vm

In order to obtain oligosaccharides, pPGM from 2 mM phosphate medium was hydrolyzed with 0.01 N HCl (Galf:H^+, 10:1, M:M) at 100^0C for 20 minutes. Anionic substances were removed by anion exchange chromatography, and neutral substances fractionated on a gel filtration column. Figure 14 shows the elution profile of the neutral substances from a Bio Gel P4 filtration column. The average size of oligomers was determined

Table 2. Action of Exo-ß-Galactofuranosidase on Various Oligosaccharides

substrate	Total CH_2O	NRT	galactose released
	mM		%
pP_2GM^{ii}	30	6.5	73
$pP_{30}GM^{ii}$	40	7.3	30
1-O-ß-M-D-Galf	50	50	100
ß-(1-->5)Galf-oligosaccharide			
$(Galf)_{6-5}$	5	0.9	100
$(Galf)_{4-3}$	5.5	1.7	100
p-Nitrophenyl-α-galactopyranoside	42	42	<0.01
p-Nitrophenyl-ß-galactopyranoside	43	43	<0.01

Substrates at indicated concentrations in an assay volume of 1 ml of buffer (66 mM acetate, pH 4.0) containing 0.7 to 6 μg of enzyme preparation were incubated at 40°C for various times. Galactose released was determined by galactose oxidase assay and total carbohydrate was determined by the phenol-sulfuric acid method.

Abbreviations: -NRT: non reducing terminal galactofuranosyl residues; -CH_2O: total carbohydrate; -$Galf_{6-5}$: mixture of hexamer and pentamer of galactofurano-oligosaccharides; -$Galf_{4-3}$: mixture of tetramer and trimer of galactofurano-oligosaccharides; 1-O-ß-M-D-Galf: 1-O-ß-Methyl-D-galactofuranoside.

Table 3. Analysis of pPGMs Before and After Treatment with Exo-ß-Galactofuranosidase

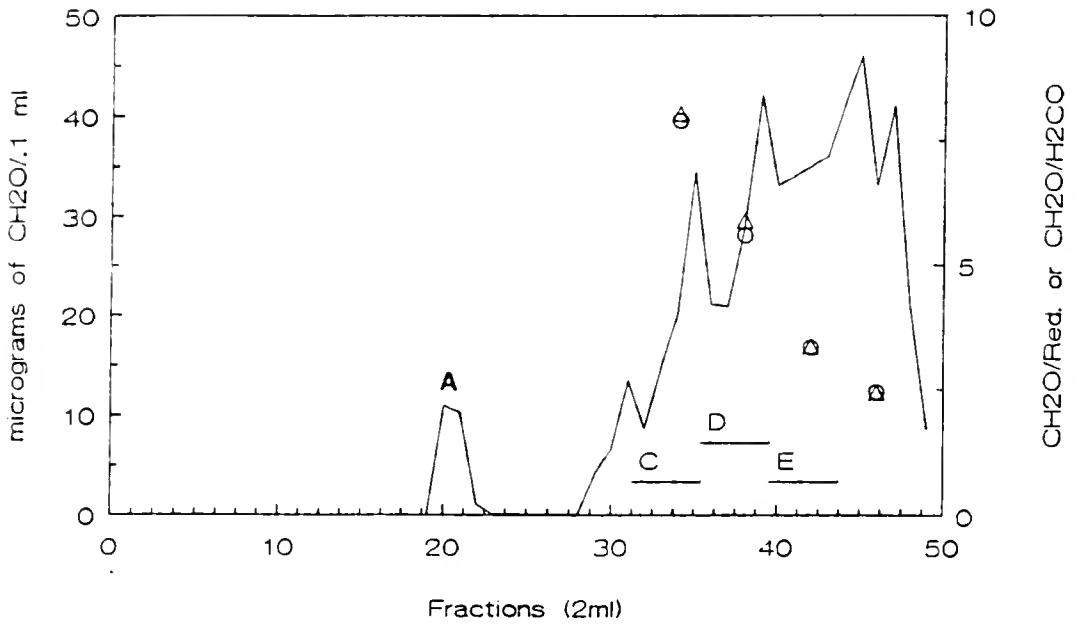
	$pP_{30}GM^{ii}$		pP_2GM^{ii}	
	Exo-ß-galactofuranosidase treatment			
	(-)	(+)	(-)	(+)
	Ratio			
Gal/CH_2O	0.55	0.37	0.6	0.16
Gal/H_2CO	6.0	3.7	5.4	1.3
$CH_2O/PO4$	10	9.2	166	109
Gal/PO_4	5.5	3.4	100	17
PO_4/H_2CO	1.1	1.1	0.05	0.08

Reaction mixture with 4 to 8 μmoles of non-reducing terminal galactofuranosyl residues in 1.5 ml of 66 mM acetate buffer containing 6 μg of purified enzyme was incubated in a 1 dm cuvette in a Jasco DP polarimeter and allowed to proceed for several hours at room temperature. The galactose released by the action of the enzyme was removed by dialyzing through a spectrapor membrane (3500 MW cut off). The retentate was used for quantitative determination. Total carbohydrate was determined by the phenol sulfuric acid method. Galactose was determined by the galactose oxidase assay. Phosphate was determined by the Ames test and formaldehyde was determined by chromotropic acid method after oxidation with periodate.

Abbreviations: CH_2O-total carbohydrate; Gal.-galactose; H_2CO-formaldehyde; PO_4- total phosphate. .

Figure 14. Gel Filtration of Oligosaccharides Obtained by Mild Acid Hydrolysis of pP_2GM^{ii}

A 400 mg sample of pP_2GM^{ii}, containing 2.5 μmoles of hexosyl residues, was treated with 2ml of 0.01 N HCl for 20 minutes as described under "Materials and Methods". The neutral mono- and oligo-saccharides were separated on a Bio-Gel P4 (200-400 mesh, Bio-Rad) at room temperature with distilled deionized water as the eluant at a flow rate of 10 ml/hour. Fractions of 2 ml were collected. Analysis of carbohydrate in each fraction was made using the phenol-sulfuric acid method. The void volume of the column corresponds to the elution position of peak A. Peaks C through E were assayed for reducing sugars using the Nelson'test for reducing sugars.


micrograms of CH2O/.1 ml
CH2O/Red. or CH2O/H2CO
Fractions (2ml)
A
C
D
E
0
10
20
30
40
50
0
5
10

calculating the ratio of total carbohydrate to reducing carbohydrate in each peak and that of total carbohydrate to formaldehyde after periodate oxidation. Fractions 33 to 36, a mixture of heptamer and septamer, were collected as peak C. Fractions 37 to 40, a mixture of hexamer and pentamer, were collected as peak D. Fractions 41 to 44 , a mixture of tetramer and trimer, were collected as peak E. Peak D (mixture of hexamer and pentamer) and peak E (mixture of tetramer and trimer) as well as pP_2GM, $pP_{30}GM$ and 1-O-ß-methyl-D-galactofuranoside were used as substrates to determine Km and Vm using Cornish-Bowden plot as described in "Materials and Methods". The values of kinetic parameters for each substrate tested are summarized in Table 4. Sample Cornish-Bowden plots are given in the Appendix. The plot of the initial reaction rate of exo-ß-galactofuranosidase on different substrates is presented in Figure 15.

The activity of purified exo-ß-galactofuranosidase toward p-nitrophenyl phosphate, p-nitrophenyl phosphocholine and bis-(p-nitrophenyl)-phosphate showed that the enzyme preparation has negligible activity ($<0.01\%$) on these substrates compared to that for 1-O-ß-methyl-D-galactofuranosidase (100%). 1-O-ß-methyl-D-galactofuranosidase hydrolysis rate was 7.6 μmoles/minute (Table 5).

Table 4. Kinetic Constants for Hydrolysis of Various Substrates by Exo-ß-Galactofuranosidase

Substrate	K_m mM	V_m mM.hr^{-1}	Specific activity μM.hr^{-1}.mg^{-1}	k_{cat} min^{-1} x10^3	k_{cat}/K_m min^{-1}.mM^{-1}
pP_2GM^{ii}	0.8	1.25	417	0.53	660
$pP_{30}GM^{ii}$	1.6	0.4	134	0.17	110
$(Galf)_{6-5}$	0.1	0.84	1200	1.5	15,000
$(Galf)_{4-3}$	0.25	0.88	1260	1.6	6,400
1-O-M-Galf.	2.65	0.6	857	1.1	415

Abbreviations: $(Galf)_{6-5}$-mixture of hexa- and penta-ß-(1-->5)-D-linked galactofurano-oligosaccharides; $(Galf)_{4-3}$-mixture of tetra- and tri-ß-(1-->5)-D-linked galactofurano-oligosaccharides; 1-O-M-Galf.-1-O-ß-methyl-D-galactofuranoside

Figure 15. Plot of Initial Reaction Rate vs Substrate Concentration

A reaction mixture consisting of substrate preparations (920 μl) containing 0.025 to 11 μmoles of non-reducing terminal galactofuranosyl residues and 80 μl of enzyme preparation (0.7 to 3 μg of protein) was incubated at 40^0C. Samples were taken out after 15, 30, 60, 120, 180 and 240 minutes. Galactose released was estimated by the coupled oxidation of galactose and o-cresol by galactose oxidase and peroxidase respectively. Initial reaction rates were determined by extrapolating to time zero. Graphs A, B, C and D are ploted on lower X axis. Graph E is ploted on the upper X axis.

Substrate designations:

A....$pP_{30}GM^{ii}$

B....pP_2GM^{ii}

C....$Galf_{6\text{-}5}$ mixture of hexa- and penta-ß-(1-->5)-galactofurano-oligasaccharides

D....$Galf_{4\text{-}3}$ mixture of tetra- and tri-ß-(1-->5)-galactofurano-oligosaccharides

E....1-O-ß-methyl-D-galactofuranoside

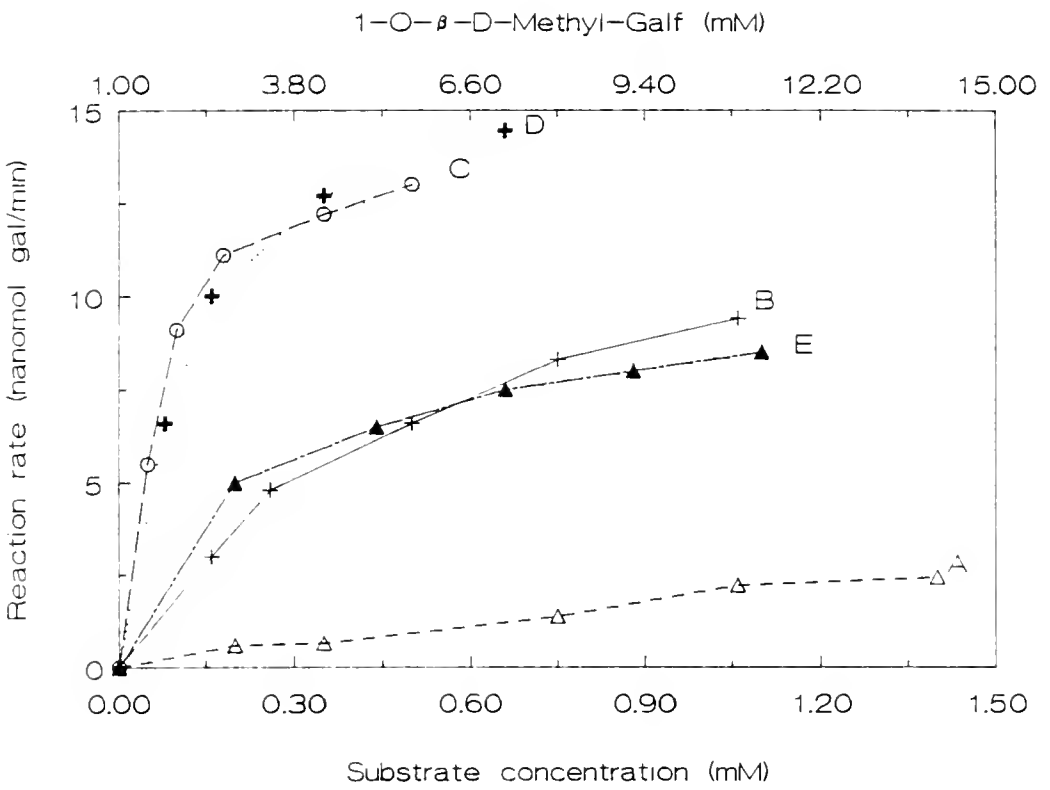

1-O-β-D-Methyl-Galf (mM)
1.00
3.80
6.60
9.40
12.20
15.00
15
10
5
0
D
C
B
E
A
Reaction rate (nanomol gal/min)
0.00
0.30
0.60
0.90
1.20
1.50
Substrate concentration (mM)

Table 5. Activities of Purified Exo-ß-Galactofuranosidase Preparation Toward Substrates for Phospho Mono- and Diesterases, and Other Glycosidases

substrate	concentration	relative hydrolysis*
	mM	%
Methyl-D-ß-galactofuranoside	50	100
p-Nitrophenyl-α-galactopyranoside	42	<0.01
p-Nitrophenyl-ß-galactopyranoside	43	<0.01
p-Nitrophenyl-N-acetyl ß-D-glucosamine	41	<0.01
p-Nitrophenyl phosphocholine	38	<0.01
p-Nitrophenyl phosphate	55	<0.01
Bis (p-nitrophenyl) phosphate	40	<0.01

*Based on that for 1-O-ß-methyl-D-galactofuranoside as 100 %. 1-O-ß-methyl-D-galactofuranoside hydrolysis rate was 7.6 μmoles/min, using 0.6 μg of protein in a total reaction mixture of 1.0 ml at pH 4.0 in 66 mM acetate buffer and at 40^0C.

DISCUSSION

Partial purification of exo-ß-galactofuranosidase from *Penicillium charlesii* in one step on a Sepharose 4B-pPGM affinity column was reported by Rietschel-Berst et al. (1977). However, additional attempts to purify the exo-ß-galactofuranosidase preparation to homogeity were unsuccessful due to the presence of at least two proteases in the culture filtrate (Pletcher, 1979; Abbas, 1987).

Exo-ß-galactofuranosidase isolated from 18-20 days culture filtrate of *Penicillium charlesii* was purified to near homogeneity, as judged by SDS and isoelectric focusing PAGE, using a variety of chromatographic methods. As described in the Results section and in Table 6, this enzyme has a molecular weight of 75 kd (Figure 6) and may exist in monomeric and/or dimeric forms (70 Kd and 150 kd, respectively) as suggested by SDS and nondenaturating PAGE. The apparent M_r of 75,000 for this enzyme is close to the value of 77,000 previously reported for the endo-ß-galactofuranase from *Penicillium oxalicum* by Reyes et al. (1992), but it is much larger than that of exo-ß-galactofuranosidase (35,000) isolated from commercial enzyme prepa-ration of *Trichoderma harzianum* by Van Bruggen-Van Der Lugt et al. (1992).

Table 6. Summary of Exo-ß-Galactofuranosidase Properties*

Property	Value	Technique
MW	75 kd	SDS-PAGE
	70 and 150 kd	Non-denaturating PAGE
Activity	pH 3.5 to 5.5	
	Optimum pH 4-4.5	In vitro assays with
	Temp. 25 to 60^0C	1-O-ß-Me-D-Galf#
	Optimum temp. 40^0C	as substrate
k_{cat} ($min^{-1}x10^3$)		
pP_2GM^{ii}	0.53	In vitro assays
$pP_{30}Gm^{ii}$	0.17	
1-O-ß-Me-D-Galf	1.07	
Apparent K_m (mM)		
pP_2GM^{ii}:	0.8	In vitro assays
$pP_{30}GM^{ii}$:	1.6	
1-O-ß-Me-D-Galf:	2.65	
Inactivation	pH less than 2.5	pH and temp. stability assays
	Temp. greater than 60^0C for incubations longer than 45 minutes	
Substrates	Extracellular pPGMs	In vitro assays
	Cell Wall pPGMs (?)	
	1-O-ß-Me-Galf	
	5-O-ß-D-Galf-oligo	
CH_2O	15% as mannose	paper chromatogram and phenol sulfuric acid assay

* See "Materials and Methods" as well as legends to figures and tables for details.

#Abbreviation: 1-O-ß-Me-Galf, 1-O-ß-methyl-D-galactofuranoside
5-O-ß-D-Galf-oligo: 5-O-ß-D-galactofurano-oligosaccharides

The enzyme is a glycoprotein, with 15% of the weight as carbohydrate as determined by the phenol-sulfuric acid assay. Mannose was the major sugar as determined by paper chromatography. Two-deoxyglucose, an inhibitor of glycoprotein biosysnthesis, was shown to inhibit the synthesis of galactofuranosidase (Gander and Fang, 1974).

Purified exo-ß-galactofuranosidase does not undergo α,ß elimination under alkaline conditions when treated with 0.4 N NaOH. This result and the observation that exo-ß-galactofuranosidase does not contain detectable galactose, suggest that pPGM is not a degradation product of exo-ß-galactofuranosidase. Salt (1983) proposed that pPGM in extracellular medium was derived from proteolysis of extracellular enzymes such as exo-ß-galactofuranosidase and phosphocholine phosphodiesterase. Neither enzyme contains galactofuranosyl residues.

Endo-ß-galactofuranase from *Penicillium oxalicum* is also a glycoprotein with 20% of its weight as carbohydrate containing mainly galactose (Reyes et al., 1992). The enzyme from *Penicillium charlesii* has a pI in the region of pH 4.35 which is much lower than that of endo-ß-galactofuranase from *Penicillium oxalicum* (Reyes et al., 1992), but similar to that of exo-ß-galactofuranosidase from *Trichoderma harzianum* (Van Bruggen-Van Der Lugt et al., 1992). Purified *P. charlesii* exo-ß-galactofuranosidase is active over a

narrow range of pH values (3.5 to 5.5). The optimum pH for activity of this enzyme was in the region of 4-4.5 (Figure 9A). The enzyme activity is unstable above pH 6. Exo-ß-galactofuranosidase was inactivated following incubations longer than 45 minutes at temperatures greater than 60^0C (Figure 9B) and the optimum temperature for activity was determined as approximately 40^0C.

Early attempts to stain galactofuranosidase in gels with Coomassie blue R-250 in 50% methanol, 5% acetic acid were unsuccessful (Rietschel-Berst et al., 1977). However, Pletcher (1979) was able to equally stain galactofuranosidase in gels with Coomassie blue R-250 in 50% methanol, 5% acetic acid and with Coomassie blue G-250 in 12% trichloroacetic acid. In our experiments, exo-ß-galactofuranosidase in gels were stained with Coomassie blue R-250 in 40% methanol, 10% acetic acid only after staining with siver stain or fixation in 30% trichloroacetic acid. Gels previously fixed with 30% trichloroacetic acid could be stained with silver stain.

The purified enzyme preparation was checked for contaminating activities such as phosphodiesterases and other glycohydrolases known to occur in crude filtrates, and no detectable activity toward the substrates used to measure the activity of these enzymes was found. The enzyme did not degrade p-nitrophenyl ß-galactopyranosidase, a specific substrate of ß-galactosidase.

The purified enzyme preparation was tested for its ability to hydrolyze different ß-1-->5 linked galactofuranosyl-containing oligosaccharides and polymers. These results are shown in Table 4. The enzyme was active on 1-O-ß-methyl-D-galactofuranoside from which it releases galactose. Galactose, as revealed by paper chromatogram and/or gel filtration, was also the only sugar released after 15 minutes, 30 minutes and 24 hours reaction of pP_2GM^{ii} or $pP_{30}GM^{ii}$ treated with the purified enzyme (Figures 10 and 11). The action of the enzyme must therefore be an endwise attack of the substrate molecule, acting in an exo-fashion. The recognition site for the enzyme is likely to be the terminal galactofuranosyl residue of the substrate. This hypothesis is supported by the fact that the enzyme is able to release galactose from 1-O-ß-methyl-D-galactofuranoside. If the exo-ß-galactofuranosidase preparation contained endo- as well as exo-galactofuranosidase activity, a nonlinear relationship between galactose released (change in observed optical rotation) and time would have been observed (Figure 12) as the endo-enzyme generated additional nonreducing terminal galactofuranosyl residues available for the exo-galactofuranosidase. Since a relatively linear response was observed and no oligosaccharides were released we concluded that the preparation of purified exo-ß-galactofuranosidase not only contains negligible quantities of endo-galactofuranosidase, but that there is negligible end-product inhibition. The

purified endo-galactofuranase from *Penicillium oxalicum* hydrolyzes internal ß-(1-->5) galactofuran linkages in homo- and hetero-polysaccharides with production of mono-di- and tri-saccharides (Reyes et al., 1992).

Recently, an exo-ß-galactofuranosidase was partially purified from commercial enzyme preparations of *Trichoderma harzianum* by using ion-exchange and adsorption columns (Van Bruggen-Van Der Lugt et al., 1992). This enzyme preparation was shown to contain some glucanase activity. The influence of pH on the activity of this exo-ß-galactofuranosidase appeared to be similar to that of the purified exo-ß-galactofuranosidase from *Penicillium charlesii*. Its optimum temperature for activity also appeared to be similar. However the molecular weight was much less than that of exo-ß-galactofuranosidase from *Penicillium charlesii*. Exo-ß-galactofuranosidase from *Trichoderma harzianum* has a very low activity on synthetic p-nitrophenyl-ß-galactofuranoside, indicating the need for more than one galactofuranose residue for binding. Treatment of extracellular polysaccharides of *Penicillium* and *Aspergillus* with the purified exo-ß-galactofuranosidase from *Trichoderma harzianum* resulted in the release of approximately 30% of the total galactose content, with the enzyme stopping near the branch point.

The rate of release of galactose from $pP_{30}GM^{ii}$ is about 5-fold less than that from pP_2GM^{ii} (Figure 14). These two species of pPGMs have almost the

same percentage of galactose and differ only by the number of phosphodiesters residues linked to the galactofuran chains. This suggests that the substitution of galactofuranosyl residues at C-6 with phosphodiesters, protects these residues from the action of exo-ß-galactofuranosidase. The slow rate of hydrolysis of $pP_{30}GM^{ii}$ may also be due to the low affinity of the enzyme for the galactofuran that have some galactosyl residues substituted with phosphodiester components. There may be a charge repulsion between the charged anionic groups of the enzyme and those of the phosphodiesters or of the N-peptidyl-ethanolamines. Alternatively, the phosphoryl residues which are attached to the galactofuranosyl units may provide either steric or electronic inhibitory effect on the catalytic process. Van Bruggen-Van Der Lugt et al. (1992) showed that partially purified *T. harzianum* exo-ß-galactofuranosidase stopped at branched ß-1-->6 of the extracellular polysaccharides of *Penicillium* and *Aspergillus*. As earlier, peptidophosphogalactomannans of *Penicillium charlesii* are substituted at C-6 of galactofuranosyl residues with phosphocholine and/or N-peptidyl phosphoryl ethanolamine. There was not phosphorylated species detectable either on paper chromatogram or by gel filtration of $pP_{30}GM^{ii}$ treated with the exo-ß-galactofuranosidase, suggesting that the enzyme does not cleave phosphorylated galactofuranosyl residues nor does it cleave interior (in relation to substituted galactofuranosyl residues)

ß-(1-->5) linked galactofuranosyl residues. What Van Bruggen-Van Der Lugt et al. (1992) considered to be a branched ß-(1-->6) galactofuranosyl chains, may be a galactofuranosyl residue substituted at C-6 with phosphodiesters components. Methylation analysis would show galactofuranosyl C-6 residues to be derivatized.

The observation that exo-ß-galactofuranosidase from *Penicillium charlesii* does not cleave phosphorylated galactofuranosyl residues can also be derived from the quantitative characterization of $pP_{30}GM^{ii}$ before and after treatment with exo-ß-galactofuranosidase (Table 3). The molar ratio of total phosphate to formaldehyde (nonreducing terminal galactofuranosyl) is the same for treated and untreated $pP_{30}GM^{ii}$ showing that the number of phosphorylated susbstitutions is the same per chain for both pPGMs. If this ratio was to decrease then it would have been an indication that the action of the enzyme released phosphorylated species. Also, the molar ratio of galactose to total phosphate has diminished indicating the release of galactose with no release of phosphate. Treatment of $pP_{30}GM^{ii}$ with exo-ß-galactofuranosidase decreased the average of galactose residues per galactan chain from 6 to 3.5, showing that an average of 2.5 galactose residues are cleaved per chain. This suggests that galactofuranosyl residues substituted at C-6 are located approximately two residues from the nonreducing terminal galactofuranosyl units.

On the other hand, for pP_2GM^{ii}, the molar ratio of phosphate to formaldehyde is larger for the treated sample than that of the untreated sample, indicating that some galactofuran chains have been completely degraded. Also here the molar ratio of galactose to phosphate decreases nearly 6-fold, confirming that there is hydrolysis of galactosyl residues but not that of phosphodiester residues. Treatment of pP_2GM^{ii} with galactofuranosidase decreased the average number of galactose residues per galactan chain from 5.4 to 1.3. This shows that there is an average of less than 2 galactose residues remaining per chain. Rietschel-Berst et al. (1977) have noted that the rate of hydrolysis of galactofuranosyl residues was rapid until there is an average of two residues per chain remaining. Gander et al. (1974) have noted earlier that pPGMs obtained from 10-day cultures contain an average of 2 galactofuranosyl residues per galactan chain.

Extracellular and cell wall polysaccharides of *Penicillium* and *Aspergillus* have been shown to be immunogenic. Galactofuranosyl residues linked ß-(1-->5) have been reported to be immunodominant in *Penicillium* and *Aspergillus* (Preston et al., 1970; Bennett et al., 1985; Notermans et al., 1988; Tuekam, 1991). The epitope degrading potential of this exo-ß-galactofuranosidase enable specific application of this enzyme. It can be useful for the detection of false positive in immunological methods such as ELISA,

and by providing an alternative to the use of synthetic ß-(1-->5) linked galactofuranoside epitopes as inhibitors. Also antibodies may be raised against this enzyme , providing an alternative for the detection of organisms that produce ß-(1-->5) linked galactofuranosyl residues. This enzyme must be taken into consideration when testing for ß-(1-->5) linked galactofuranosyl epitopes. A negative test may be due to a very active exo-ß-galactofuranosidase present in the sample which has degraded the epitopes rendering them undetectable by the test system used. However it must be recognized that exo-ß-galactofuranosidase has little activity on phosphodiester-containing glycopeptides.

The structures of *Penicillium* membrane-bound and extracellular pPGMs are very complex. Their roles, other than providing a barrier between the fungal cytoplasm and its environment, and providing a nutrient source (Gander and Laybourn, 1981), are mostly unknown. However, a likely role is that of a temporary storage form of phosphate, carbohydrate, amino acids and phosphatide components. Phosphorylation of galactofuranosyl residues may be a way to control depolymerization of these pPGMs, so that the enzyme only cleaves what is needed. A phosphodiesterase and/or a phosphomonoesterase may then remove the phosphate, thereby allowing the galactofuranosidase to resume hydrolysis. This hypothesis needs further testing using purified enzyme

preparations to determine the rate at which extracellular *Penicillium* phosphodiesterases, acid phosphomonoesterase and exo-ß-galactofuranosidase catalyzes hydrolysis of pPGM, cell wall phosphodiesters and galactofuranosyl residues when the enzymes are added singly and in combinations to these potantial substrates.

Exo-ß-galactofuranosidase specifity and mode of action should be a useful tool for analysing the fine structures of polysaccharides. The physiology and biochemistry which occurs as the fungi survive and continue to grow in a nutritionally unbalanced environment is only partially understood. The sequence of events and the inhibitory or synergistic interacting species that participate in regulating the lysis process in fungi is largely unknown. Purified exo-ß-galactofuranosidase as well as other purified lytic enzymes produced by *Penicillium charlesii*, may help determine if pPGM influences several other physiological processes such as buffering of the acidity at the cytoplasmic membrane-cell wall interface, and modulating the osmolarity over a range of concentrations of nutrients.

CONCLUSION

The major objectives of this investigation were to purify *Penicillium charlesii* exo-ß-galactofuranosidase to homogeneity and to determine its properties regarding (i) its composition as compared with peptidophospho-galactomannan, and (ii) its activity on peptidophosphogalactomannan containing galactofuranosyl residues substituted at C-6 with phosphodiester residues.

The enzyme was purified to homogeneity. It was shown that the highly phosphorylated glycopeptides of *Penicillium* are 6-fold poorer substrates for exo-ß-galactofuranosidase than non-phosphorylated glycopeptides. I conclude from this data coupled with that obtained previously that depolymerization of *Penicillium* peptidophosphogalactomannan galactofuran chains is regulated by the extent of substitution with phosphodiester residues. This conclusion is consistent with the 30-fold increase in glycerol-3-phosphocholine phospho-diesterase activity observed by others in this laboratory when *Penicillium charlesii* is cultured on low phosphate medium. These cultures resulted in a peptidophosphogalactomannan which contained about 15-fold less phosphate and one in which galactofuranosyl chains were rapidly depolymerized.

SUMMARY

1. Exo-ß-galactofuranosidase was isolated and purified to homogeneity from concentrated cultures filtrates of *Penicillium charlesii* by a five-step procedure involving ion exchange and gel filtration chromatography. The homogeneity of the purified galactofuranosidase was established using SDS-PAGE and isoelectric focusing PAGE. The enzyme contains 15% carbohydrate; mannose is the primary sugar and no galactose was detected.
2. The general properties of exo-ß-galactofuranosidase, pH and temperature optima, pH and temperature stability, specificity and kinetic properties, were determined using in vitro assays.
3. Experiments were performed to determine the influence of phosphodiesters on the activity of exo-ß-galactofuranosidase on peptidophospho-galactomannan. The results of these experiments indicated that phosphate on the galactofuran chain decreases exo-ß-galactofurano-sidase activity by nearly 6-fold.
4. Exo-ß-galactofuranosidase does not cleave galactofuranosyl residues substituted at C-6 with phosphodiester components.

APPENDIX

Table 7. Czapek-Dox Agar

Nutrients	Constituents	mM	g/l
Carbon	Glucose	278	50
Nitrogen/Sodium	$NaNO_3$	23.5	2
Phosphorus/Potassium	KH_2PO_4	7.4	1
Sulfur/Magnesium	$MgSO_4.7H_2O$	4.1	1
Potassium	KCl	6.7	0.5
Sulfur/Iron	$FeSO_4.7H_2O$	0.06×10^{-3}	0.018
Agar	--	--	20

_Glucose is autoclaved in d.d. water; other constituents are autoclaved in tap water. The above mixed and dispensed into cultures tubes as soon as the flasks can be handled.

Table 8. Raulin-Thom Medium

Nutrients	Constituents	Concentration	
		mM	g/l
Carbon	Glucose	278	50
	Tartaric Acid	17.8	2.7
Carbon/Nitrogen	Ammonium Tartrate	14.5	2.7
Nitrogen/Phosphorus	$(NH_4)_2HPO_4$	20.2	2.7
Nitrogen/sulfur	$(NH_4)_2SO_4$	1.3	0.17
Potassium	K_2CO_3	2.9	0.4
Magnesium	$MgCO_3$	3.4	0.29
Zinc	$ZnSO_4.7H_2O$	0.16×10^{-3}	0.05
Iron	$FeSO_4.7H_2O$	0.17×10^{-3}	0.05

Glucose is autoclaved in d.d. water separately from other constituents which are autoclaved in equal vo;ume of tap water (110^0C, 25 minutes). After cooling to room temperature, the above are mixed thoroughly by swirling and then dispensed in 150-200 ml volumes into cultures flasks.

Table 9. Standard Growth Medium (SG)

Nutrients	Constituents	Concentration	
Macronutrients		mM	g/l
Carbon	Glucose	320	57.6
Nitrogen	NH_4Cl	80	4.3
Phosphorus	Na_2HPO_4	20	2.8
Sulfur	Na_2SO_4	2	0.28
Buffer/Chelator	Na citrate	12.5	3
	Citric acid	12.5	2.2
Potassium	K_2CO_3	3	0.42
Magnesium	$MgCO_3$	4	0.34
	NaCl	20	1.2
Micronutrients		element, ppm	salt mg/l
Iron	$FeSO_4.7H_2O$	10	50
Zinc	$ZnCl_2$	5	10.4
Manganese	$MnCl_2.4H_2O$	5	18.0
Molybdenum	$(NH_4)_6(MO_7O_{24}.4H_2O$	2	3.7
Calcium	$CaCl_2.5H_2O$	0.5	1.8
Copper	$CuSO_4.5H_2O$	0.4	1.6
Vanadium	NH_4VO_3	0.2	0.46
Boron	H_3BO_3	0.1	0.57
Chromium	$Cr_2(SO_4)_3.12H_2O$	0.1	0.93
Nickel	$NiCl_2.6H_2O$	0.1	0.4
Colbalt	$CoCl_2.6H_2O$	0.1	0.4

Micronutrients, except for $FeSO_4$, were stored as individual 4000-fold concentrated stock solutions. Before autoclaving, 0.25ml/L final volume of each and solid $FeSO_4$ were added to diluted salts solution. Carbon source and combined salts were separately dissolved in deionized distilled water, autoclaved at 121^0C for 25 minutes and combined aseptically after cooling. The pH of combined medium was 5.95.

Scheme I. Isolation and Fractionation of Extracellular Glycopeptides of *Penicillium charlesii*

1. Dialyze culture filtrate versus tap water then versus distilled deionized water at 4^0C.

2. Add 5% by volume 1 M pH 7.0 sodium borate, then 4% by volume of 5% cetyltrimethylammonium bromide (CETAB). With stirring, adjust pH to 9.0 with 1.0-10 N NaOH. Decant supernatant liquid and discard.

3. Redissolve gummy precipitate in minimal volume of 0.5 N NaCl

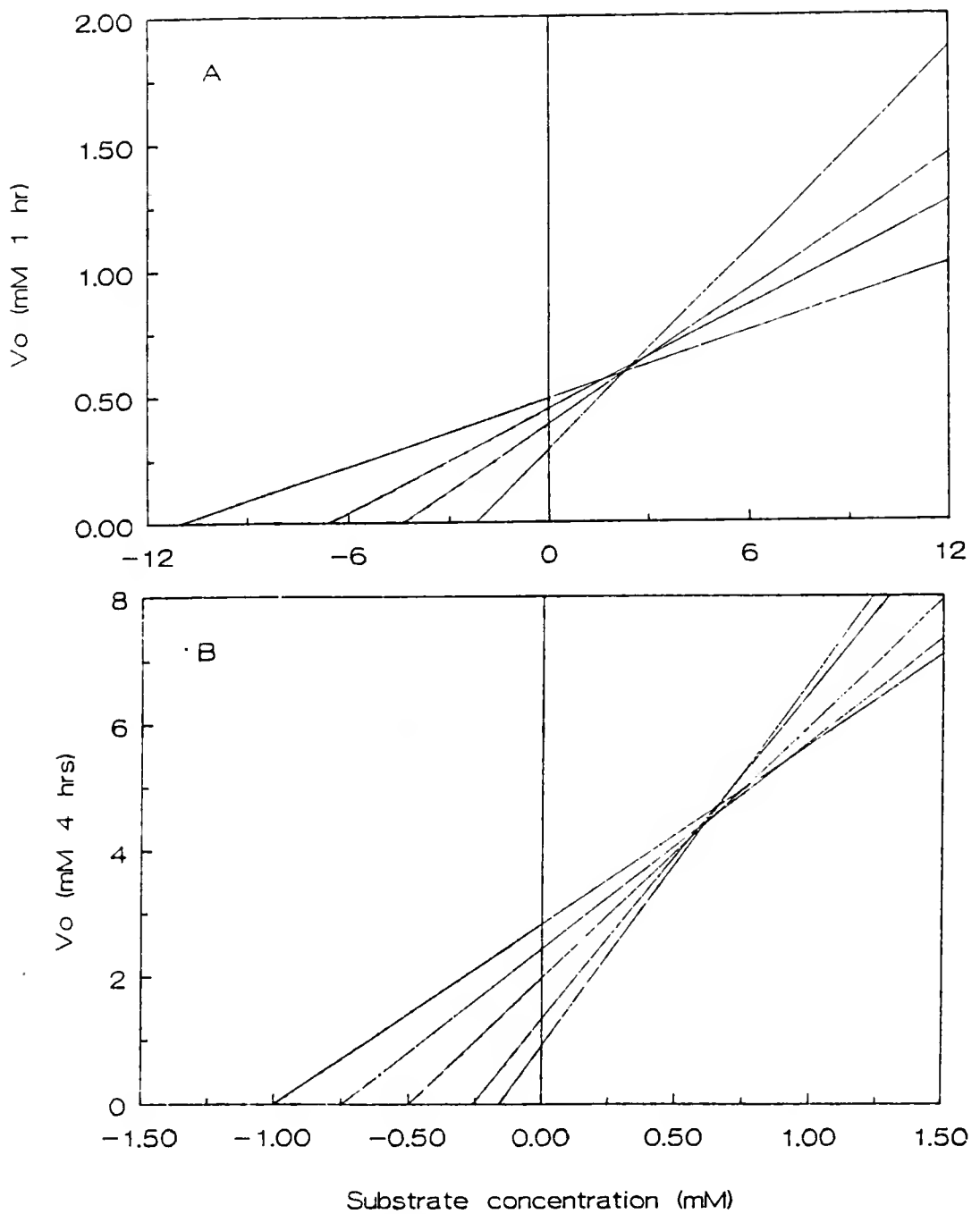
4. Reprecipitate with 3 volumes of ethanol. Centrifuge and discard supernatant. Wash pellet two times with ethanol.

5. Decant ethanol and redissolve precipitate in 50 mM $K_2B_4O_7$. Filter and discard insoluble material.

6. Apply to Whatman DEAE Cellulose (DE-52) column preequilibrated with 50 mM $K_2B_4O_7$

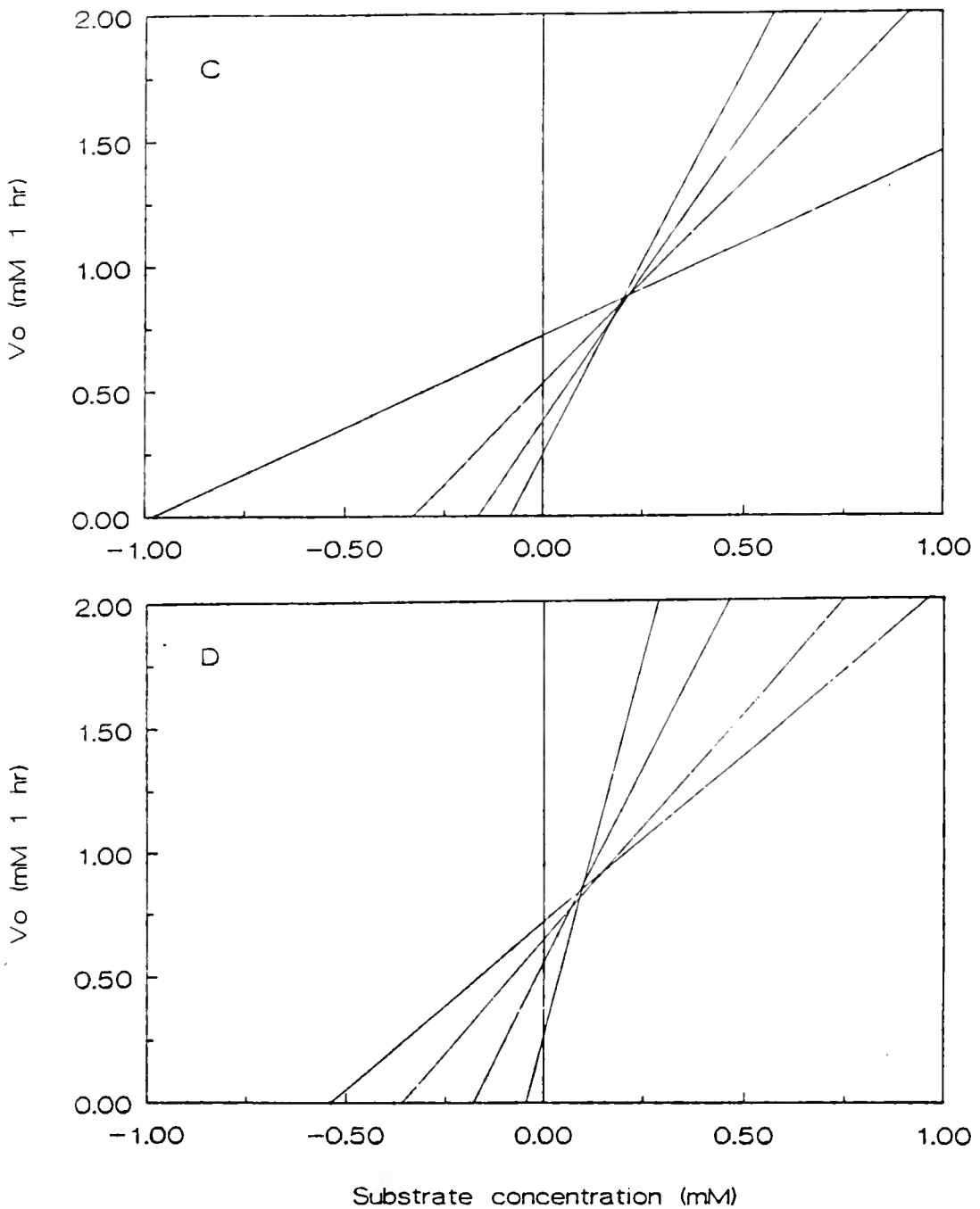
7. Wash column stepwise with 1L each of:
   a) 50 mM $K_2B_4O_7$
   b) distilled deionized water
   c) 0.01 N HCl
   d) 0.01 N HCl/0.06 N LiCl (Major glycopeptide fraction= pPGM[ii])
   e) 0.01 N HCl/0.4 N LiCl (Minor glycopeptide fraction =pPGM[iii])
   f) 0.5 N NaCl

8. Assay fractions for carbohydrate using Dubois phenol-sulfuric acid assay. Dialyze major carbohydrate containing fractions against distilled deionized water. Concentrated on rotory evaporator and redialyzed as above. Lyophilized and weighted. Store powder in vials over dessiccant until used.



Cornish-Bowden Plots A and B for Determination of Km and Vm

Panel A: 1-O-ß-methyl-D-galactofuranoside
Panel B: pP_2GM^{ii}



Cornish-Bowden Plots C and D for the Determination of Km and Vm

Panel C: $Galf_{4\text{-}3}$-mixture of tetra- and tri- galactofurano-oligosaccharides
Panel D: $Galf_{6\text{-}5}$-mixture of hexa- and penta- galactofurano-oligosaccharides.

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BIOGRAPHICAL SKETCH

Brigitte Albertine Tuekam was born in Bandjoun, Cameroon, in November 1961 as B. A. Tamdem. She attended primary and elementary school from 1966 to 1972 and obtained her certificate of achievement in 1972. From 1972 to 1980, she attended secondary school and obtained her B.E.P.C.(< = > General Certificate of Education, Ordinary Level) in 1977, her Probatoire in 1979, and her Baccalaureat (< = > G.C.E. Advanced Level) in 1980. From 1980 to 1983 she attended the University of Yaounde, Cameroon, where she graduated in 1983 with a licence (bachelor's degree) in natural sciences, major zoology. She was appointed as a professor of secondary school in Cameroon where she taught from 1983 to 1986.

She moved to Florida in 1987 and enrolled in the graduate program in the Department of Microbiology and Cell Science in August 1988. She obtained an M.S. degree from the University of Florida in 1991.

Brigitte Albertine Tuekam is married and has two daughters (Rosine Sylvia and Sandrine Nadege) and a son (Steve Cedric) with her husband Steve.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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May, 1993

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